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Control of T lymphocyte responses by CD95-mediated apoptosis

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CONTROL OF T LYMPHOCYTE RESPONSES BY CD95-MEDIATED APOPTOSIS

Submitted by Lucy S.K. Walker

for the degree of Ph.D. at the University of Bath

1997

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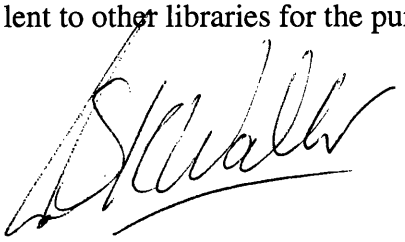

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This thesis is dedicated to
Kathleen Paskin

Acknowledgements

I gratefully acknowledge all those who have made this project possible including my supervisors Dr David Sansom and Dr Nick Hall and The Wellcome Trust.

Thanks go to all at BIRD for making my time in Bath so special, particularly the immunology group for showing me the true meaning of team spirit. I will remember with pleasure Dr Martina Boshell, Dr Anne Wilson, Dr Chris Edmead, Declan O'Byrne, Dr Yusuf Patel and in particular Dr Julie McLeod for being the fount of all laboratory knowledge, George Boulougouris for putting up with my teasing and Claire Ellwood for keeping us all in order.

Special thanks go to Dr David Sansom and Dr Julie McLeod for endless encouragement, support and valuable career advise. I have listened and learnt.

I would also like to thank my family for all their support, and my flat-mates Jessica Sweett, Stacey Lillington, Alex Wilson, Sue Walters, and Mark Ellison for making it fun.

CONTENTS

SUMMARY

T cells comprise a key component of the immune response and aberrant T cell function underlies a number of high profile medical conditions including acquired immune deficiency syndrome and autoimmune diseases. Consequently, a better understanding of the interactions which control T cell responses has significant clinical implications. Recent interest has focused on the role of apoptosis in the control of T cell responses and in particular the involvement of the Fas receptor (CD95) in this process. This study investigated the role of CD95-mediated apoptosis in human T cell responses, with a view to determining how the expression of CD95 and its ligand (CD95L) was regulated during the T cell response to antigen engagement and the consequences of this for apoptosis induction. In particular, the role of the CD95 pathway in activation-induced cell death following antigen restimulation was addressed since this is believed to represent an important physiological trigger for CD95-mediated killing. Finally, the modulation of CD95-induced apoptosis by additional receptor:ligand interactions was examined in order to assess how the context of CD95 engagement affected the outcome. In contrast to previous studies, the data presented here demonstrate that, unlike transformed or cloned T cell lines, normal activated human T cells were largely resistant to CD95-mediated apoptosis when stimulated with anti-CD95 antibodies or when subjected to TCR restimulation. This was found to be the case despite expression of both CD95 and CD95L. Since provision of signalling via both the TCR CD3 component and the costimulatory receptor CD28 further inhibited the apoptotic response to CD95 ligation, it is postulated that "correct" activation of T cells via these receptors may confer resistance to CD95-mediated apoptosis. Conversely, T cells which were activated non-specifically by supernatant transfer exhibited greater sensitivity to CD95-induced death. Thus, a major role for CD95-mediated apoptosis may be the elimination of potentially autoreactive "bystander" T cells.

Abbreviations

Ab	Antibody
AICD	Activation Induced Cell Death
ALPS	Autoimmune Lymphoproliferative Syndrome
AM	Acetoxymethyl
AP-1	Activator Protein 1
AP-2	Adapter Protein 2
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
Ca^{2+}	Calcium ions
CAPK	Ceramide-Activated Protein Kinase
CAPP	Ceramide-Activated Protein Phosphatase
CD	Cluster of Differentiation
cDNA	complementary Deoxyribonucleic Acid
CD95L	CD95 Ligand
ced genes	*Caenorhabditis elegans* death genes
CHO cells	Chinese Hamster Ovary cells
CLARP	Caspase-Like Apoptosis Regulatory Protein
CO_2	Carbon Dioxide
ConA	Concanavalin A
CPM	Counts Per Minute
CTL	Cytotoxic T Lymphocyte
CTLA4	Cytotoxic Lymphocyte Associated Antigen 4
ddH_2O	double distilled H_2O
DAG	1,2 Diacylglycerol
DED	Death Effector Domain
DEPC	Diethylpyrocarbonate
DISC	Death Inducing Signalling Complex
DMEM	Dulbecco's Minimal Essential Medium
DNA	Deoxyribonucleic Acid
DR-3/4	Death Receptor 3/4
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter
FADD	Fas Associated Death Domain containing protein

FAP1	Fas Associated Phosphatase 1
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FLICE	FADD-like ICE
FLIPS	FLICE Inhibitory Proteins
FSC	Forward Light Scatter
h	Hour(s)
HBSS	Hank's Balanced Salt Solution
^{3}H-thymidine	Tritiated thymidine
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICAM	Intercellular Adhesion Molecule
ICE	Interleukin-1β Converting Enzyme
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 Receptor
IP3	Inositol 1,4,5-trisphosphate
IU	International Units
ITAM	Immunoreceptor Tyrosine Based Activation Motif
JNK	c-Jun N-terminal Kinase
kDa	Kilodaltons
LFA	Lymphocyte Function-Associated Antigen
LPS	Lipopolysaccharide
$\Delta\Psi_m$	Mitochondrial Transmembrane Potential
mAb	monoclonal Antibody
MAPK	Mitogen Activated Protein Kinase
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
min	minute(s)
Mls	Minor Lymphocyte-stimulating antigen
mRNA	Messenger Ribonucleic Acid
NFκB	Nuclear Factor κB
NGFR	Nerve Growth Factor Receptor
NOD	Non Obese Diabetic
PARP	Poly(ADP-Ribose) Polymerase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PHA	Phytohaemagglutinin

PE	Phycoerythrin
PI	Propidium Iodide
P/I	PMA/Ionomycin
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidlyinositol 4,5 bisphosphate
PKB/C	Protein Kinase B/C
PLC	Phospholipase C
PMA	Phorbol Myristate Acetate
PS	Phosphatidylserine
RNAse	Ribonuclease
SDS	Sodium Dodecyl Sulphate
SEB	Staphylococcal Enterotoxin B
SEM	Standard Error of the Mean
SH	*src* Homology
SLAM	Signaling Lymphocytic Activation Molecule
SLE	Systemic Lupus Erythematosus
SSC	Saturated Sodium Chloride
TCR	T Cell Receptor
Th	T helper
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
VLA	Very Late Antigen
Z-VAD-FMK	Cbz-Val-Ala-Asp(OMe)-fluoromethyl ketone

CHAPTER 1

Introduction

1.1 IMMUNITY VERSUS TOLERANCE

An essential feature of the immune system is the ability to distinguish between self and non-self in order to successfully eliminate invading pathogens. To this end, a wide variety of cell types cooperate to mount a co-ordinated defence response which must be in place as rapidly as possible since pathogens frequently replicate rapidly. T cells play a key role in the induction of such a response: having recognised foreign material by the use of a specialised T cell receptor (TCR) they go on to fulfil diverse functions including cytotoxicity, the facilitation of B cell antibody production and the recruitment of accessory cells which together act to eliminate the pathogen. However, the effector mechanisms of the immune system may also pose a considerable threat to the host and therefore controlled initiation and appropriate termination of immune responses are of paramount importance to avoid destruction of self tissue. In order to achieve this regulation, cells of the immune system have evolved stringent activation requirements such that a co-ordinated set of signals must be provided in order for productive activation to occur. Furthermore, the existence of an intrinsic death programme which can be receptor-driven potentially allows the controlled elimination of cells which have fulfilled their immune function or which may be dangerous to the host. The mechanisms of selective activation or apoptosis of T cells can thus be used to control the response to antigen engagement in order to ensure reactivity against invading pathogens (immunity) and yet to maintain non-responsiveness (tolerance) against self tissue. The importance of controlled cell deletion within the immune system has become increasingly apparent, and this study therefore sought to examine the process of CD95-mediated apoptosis as one mechanism of peripheral T cell tolerance. Since the signals which mediate T cell activation also contribute to subsequent T cell survival, this process therefore has a direct bearing on the control of apoptosis induction. Thus a review of current

understanding of the interactions controlling the generation of both T cell immunity and T cell tolerance follows.

1.2 T CELL IMMUNITY

1.2.1 T Cell Repertoire

In order to create the scope for recognising a wide range of foreign antigens, T cells generate the potential for global reactivity yet maintain selective non-responsiveness against self by the use of tolerance mechanisms. This generation of diversity is achieved at the molecular level by gene rearrangement processes (Chien et al., 1984) such that large numbers of receptors are produced which can recognise numerous different antigens (Marrack and Kappler, 1987; Maryanski et al., 1997). The antigen receptor on T cells is the TCR, however similar mechanisms operate to generate diversity for B cell antigen receptors (which comprise surface immunoglobulin) and together these two cell types mediate specific antigen recognition and represent the "eyes" of the immune system. Having generated a vast repertoire of antigen recognition receptors, any that might be self-reactive, and might therefore trigger autoimmunity, must subsequently be screened out. This process is achieved by tolerance mechanisms which allow the immune system to selectively ignore the host's own tissue and which are reviewed in section 1.3.

1.2.2 Antigen Recognition

The recognition of antigen by T cells is restricted by the requirement for appropriate antigen presentation by cells expressing 'self' markers (Zinkernagel et

al., 1974). The cells which fulfil this function are termed antigen presenting cells (APCs) and include dendritic cells, macrophages and B cells (Ellis et al., 1991). APCs determine which antigenic epitopes are available for T cell recognition by displaying discrete peptide fragments bound to Major Histocompatibility Complex (MHC) class I and class II molecules termed Human Leukocyte Antigen (HLA) molecules in human and H-2 molecules in mouse. These molecules represent a group of highly polymorphic cell surface glycoproteins of which two classes exist and which are designed to bind peptide antigens and present them to the TCR (Babbitt et al, 1985). The peptides bound by class I molecules are generally derived from an intracellular location and are usually approximately 8-10 amino acids in length (Townsend and Bodmer, 1989; Yewdell and Bennick, 1992). In order to become associated with class I molecules, such peptides are delivered to the secretory pathway in conjunction with the transporter associated with antigen processing (TAP) complex which is itself encoded by MHC genes (Trowsdale et al., 1991; Kleijmeer et al., 1992). In addition, similar mechanisms appear to exist for peptide loading of class II molecules using HLA-DM (Morris et al., 1994).

Contrasting with class I molecules, class II molecules bind slightly larger peptides of 15-20 amino acids in length (Germain and Hendrix, 1991). An invariant chain, a non-polymorphic gene product, is also associated with the class II α and β chains and is essential for their proper assembly (Peterson and Miller, 1990; Lamb et al., 1991; Anderson and Miller, 1992). Crystalographic studies established that the structure of the HLA class I molecule HLA-A2 comprised a peptide binding groove formed by two α helices lying on a β pleated sheet (Bjorkman et al., 1987) and subsequent analysis has revealed a similar structure for class II molecules (Brown et al., 1993). Thus the T cell perceives non-self (antigen) only in the context of self (HLA).

The class of MHC molecule on which the peptide antigen is presented is important in the control of the T cell response since class I molecules interact with

a T cell co-receptor termed CD8, whilst class II molecules preferentially bind to the T cell CD4 co-receptor (Doyle and Strominger, 1987). The CD8+ subset comprise cytotoxic T cells whilst the CD4+ subset are broadly categorised as helper T cells since they play a key role in the facilitation of B cell antibody production via the secretion of cytokines such as the interleukins (IL) IL-4 and IL-5. Since HLA class I is ubiquitously expressed throughout the body, virtually all cells can present antigen to the CD8+ cytotoxic subset whereas HLA class II expression is mainly restricted to specialised cells of the immune system.

1.2.3 TCR Signalling

T cell antigen recognition is mediated by the TCR which comprises an α and β chain (or less frequently a γ and δ chain) linked by a single disulphide bridge (Marrack and Kappler, 1987) and is a member of the immunoglobulin (Ig) superfamily of receptors. Each T cell has a unique TCR$\alpha\beta$ specificity to facilitate antigen recognition. However, the $\alpha\beta$TCR itself is not believed to mediate signal transduction by virtue of its short cytoplasmic domain: rather, a separate transmembrane signalling component, CD3, comprising $\gamma\delta\varepsilon$ chains in addition to the ζ family of hetero- and homo-dimers (Marrack and Kappler, 1987; Brocker and Karjalainen, 1995), exists in close proximity to the TCR and is thought to transduce the signals initiated by the process of T cell antigen engagement (see figure 1.1) (Howard et al., 1992; Rudd et al., 1994).

Triggering of the TCR/CD3 complex initiates multiple signalling pathways including the requisite activation of intracellular protein tyrosine kinases (Klausner and Samelson, 1991). However, the TCR itself does not possess intrinsic kinase activity and instead this pathway relies on the recruitment and activation of *src* family protein tyrosine kinases such as p56^{lck}, p60^{yes}, p59^{fyn} (Klausner and Samelson, 1991). The members of this family are characterised by a common structure which includes a unique N-terminal region, *src* homology

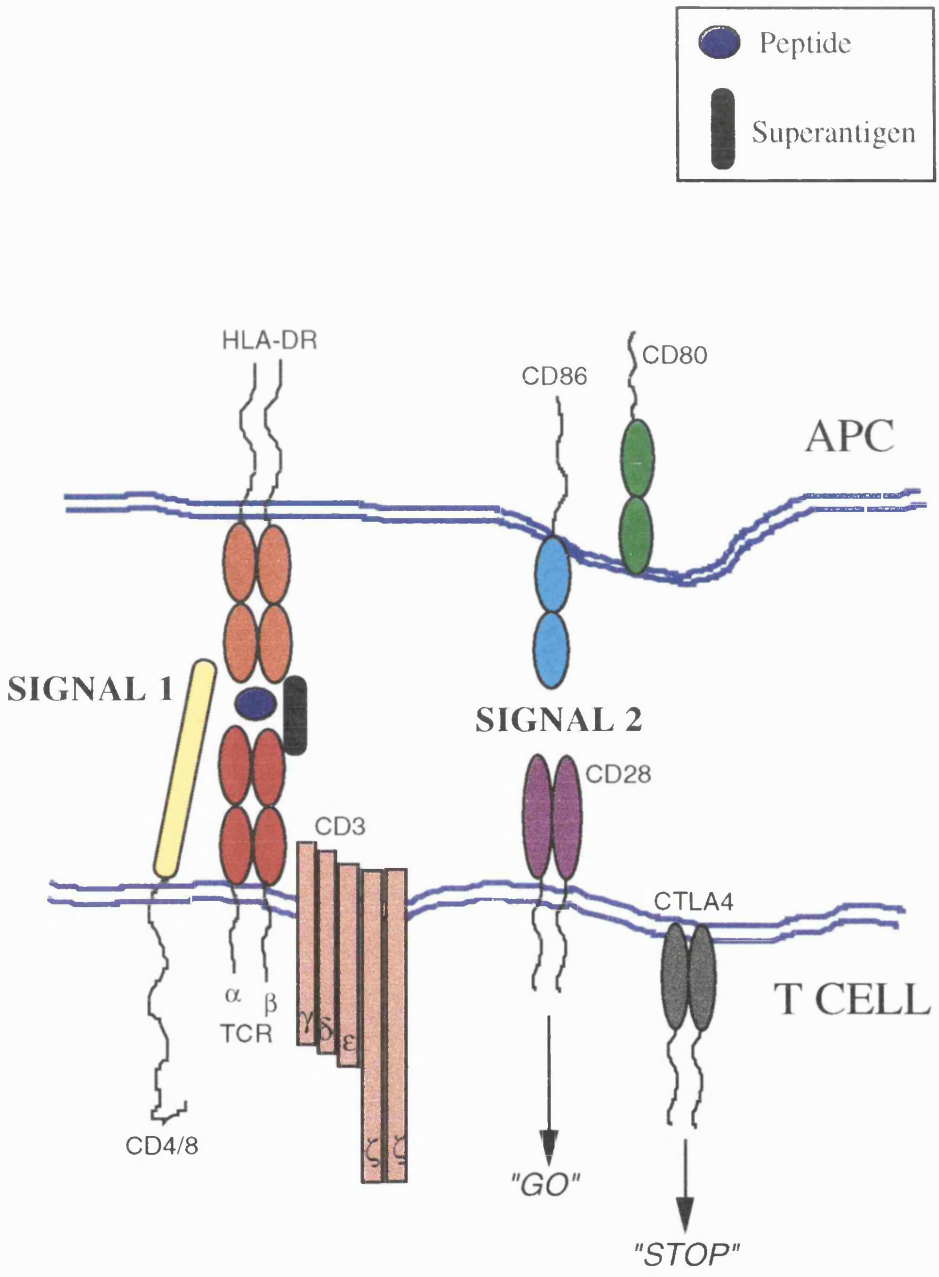


Figure 1.1: Schematic representation of antigen presentation to T cells. Signal 1 is provided by TCR engagement of the antigen in the context of HLA-DR expressed on the APC. Signal 2 (costimulation) is provided by CD28 engagement via APC CD80/86. APC = antigen presenting cell, HLA = Human Leukocyte Antigen, TCR = T cell receptor

regions 2 and 3 (SH2 and SH3 domains), a catalytic tyrosine kinase domain (SH1 domain) and a regulatory C-terminal motif (Rudd et al., 1994). Whilst p56^{lck} associates with the CD4/8 co-receptors (Rudd et al., 1994; Thome et al., 1995), p59^{fyn} interacts with the TCRζ chain and the CD3γδε chains via its N-terminal region (Rudd et al., 1994). In addition, the *syk* family tyrosine kinase ZAP-70 is recruited to the TCRζ chain and can contribute to T cell signal transduction during the antigen presentation event (Samelson and Klausner, 1992; Chan et al., 1994; Timson-Gauen et al., 1994; Chan et al., 1995; Thome et al., 1995).

The targets of the kinases associated with TCR-induced signal transduction are believed to include phospholipase Cγ (Park et al., 1991) which in turn mediates the hydrolysis of phosphatidlyinositol 4,5 bisphosphate (PIP2) yielding inositol 1,4,5 triphosphate (IP3) and 1,2-diacylglyerol (DAG) (Berridge and Irvine, 1984; Perlmutter et al., 1993). Whilst IP3 triggers calcium (Ca^{2+}) release from intracellular stores (Weiss et al., 1984), DAG activates certain isoforms of protein kinase C (PKC) (Genot et al., 1995) which can stimulate the activation of cellular proto-oncogenes such as p74^{raf} and p21^{ras} (Rudd et al., 1994). leading to mitogen activated protein kinase (MAPK) cascades (Li et al., 1996). The phorbol ester phorbol myristate acetate (PMA) can be used as a pharmacological tool to activate PKC by binding to DAG-binding sites (Kraft and Anderson, 1983).

Thus the recognition of foreign material by T cells involves the processing and displaying of peptide antigen in the context of self HLA molecules on APCs. Binding of this peptide/HLA complex to the TCR triggers T cell activation signals via the CD3 complex and ζ chains (signal 1). However, TCR ligation *per se* is not sufficient to fully activate T cells and the requirement for an additional costimulatory signal (signal 2) is reviewed in section 1.2.5. Figure 1.1 therefore illustrates the key receptors involved in T cell recognition of antigen, with the addition of CD28, a costimulatory receptor which provides a "go" signal, and its homologue CTLA4 which delivers a "stop" signal to T cells. The control of T cell

fate by receptors such as CD28 and CTLA4 are reviewed in more detail in sections 1.2.6-1.2.11.

1.2.4 Superantigens

One experimental model of T cell activation which has been exploited in this study involves the use of superantigens which differ from conventional peptide antigens by interacting with both the TCR and HLA molecules outside their specific antigen-binding areas (Herman et al., 1991) as indicated in figure 1.1. Superantigens such as Staphylococcal enterotoxins bind directly to HLA class II proteins (Buxser et al., 1981) without the need for processing, and interact with the Vβ chain of the TCR. In bypassing the specificity-determining regions, superantigens thus provide a more global stimulus than conventional peptide and activate T cells on the basis of their particular TCR-Vß chain expression (Kappler et al., 1989; Dohlsten et al., 1993) triggering the proliferation of approximately 5-40 percent of the T cell population (Janeway et al., 1989). *In vivo*, injection of superantigens induces both T cell proliferation and apoptosis highlighting the fine balance between life and death in response to activation stimuli (MacDonald et al., 1991). Superantigen stimulation has therefore been utlised *in vitro* in this study to investigate the processes of T cell activation and apoptosis.

1.2.5 T helper 1 and T helper 2 Cells

Within the CD4+ compartment, T cells can be further subdivided according to their preferential cytokine output, such that cells which predominantly produce interferon γ (IFNγ) and/or IL-2 are classed as Th1 (T helper 1) cells whilst those which secrete cytokines such as IL-4, IL-5, and IL-10 are defined as exhibiting a Th2 (T helper 2) profile (Fiorentino et al., 1989; Mosmann and Coffman, 1989; Lederer et al., 1993). The Th1/2 skewing of T cell responses influences the nature of the subsequent immune response (for example Th1 responses may favour

inflammation whilst Th2 responses may favour antibody production) and appears to have important consequences for whether a pathogen is successfully eliminated (Bass et al., 1989; Else et al., 1993; Reiner and Locksley, 1993). The differentiation of T cells towards a particular Th phenotype is largely influenced by the cytokine environment (Noble et al., 1993; Seder et al., 1993) and a large body of literature exists regarding the categorisation of cells as Th1 or Th2, and the dangers therein (Allen and Maizels, 1997; Karulin and Lehmann, 1997; Romagnani, 1997).

1.2.6 Costimulation via CD28

At the level of a single T cell, the difference between the induction of immunity or tolerance ultimately depends on whether the recognition of antigen triggers T cell activation, anergy (antigen-specific non-responsiveness) or apoptosis. This fundamental choice appears to be largely controlled by the availability of alternative receptor:ligand interactions in addition to the triggering of the antigen receptor (TCR). It is now well established that effective T cell activation requires costimulatory signals, in addition to antigen derived signals via the TCR/CD3 complex, in order for proliferation to be triggered. In the absence of such costimulation, antigen engagement may instead lead to anergy or even apoptosis (Ramensee et al., 1989; Schwartz, 1990; Jenkins et al., 1991) emphasising the importance of this interaction in the control of T cell survival. The provision of additional signals is thus a key factor in dictating the outcome of a TCR signalling event, and one molecule which is important in this regard is the CD28 receptor. CD28 has a glycosylated molecular weight of 44 kDa, and is expressed as a homodimer on the majority of CD4+ T cells and approximately 50% of CD8+ T cells (Linsley and Ledbetter, 1993; June et al., 1994). The gene for CD28 maps to chromosome 2q33-34 and encodes a 202 amino acid protein of which 41 amino acids comprise an intracellular portion responsible for signal transduction (Dariavach et al., 1988). Whilst TCR/CD3 signals alone are insufficient for T cell

activation, the simultaneous provision of CD28 receptor engagement allows T cell proliferation and IL-2 production to ensue (Gimmi et al., 1991; Jenkins et al., 1991; Linsley et al., 1991a; Razi-Wolf et al., 1992; Sansom et al., 1993). The effect on IL-2 production was found to operate via both transcriptional and post-transcription mechanisms and in addition similar effects were observed for other cytokines including IL-4, IL-8, IL-13 and TNFα (Lindsten et al., 1989; Thompson et al., 1993; June et al., 1994).

In addition to CD28 engagement, a number of other receptor:ligand interactions between the T cell and APC may be important in optimising activation, both by enhancing adhesion and possibly by playing signalling roles. The interaction of CD58 on the APC with CD2 on the T cell is believed to be important in this regard (Howard et al., 1992; Kanner et al., 1992;) and similarly adhesion molecules such as CD54, CD11a/CD18 (Damle et al., 1992; van Seventer et al., 1990) and the integrin associated protein (IAP/CD47) (Reinhold et al., 1997) promote the cell:cell contact between T cell and APC. In particular, a role for the CD95 family member 4-1BB in costimulating T cell proliferation is gaining credence and may preferentially operate in the CD8+ T cell subset (DeBenedette et al., 1997; Shuford et al., 1997) whilst the signaling lymphocytic activation molecule (SLAM) appears to offer an additional route for the promotion of T cell activation (Aversa et al., 1997).

1.2.7 Ligands for CD28

The ligands for the CD28 receptor belong to the CD80 family of proteins and are expressed on "professional" APCs of the immune system. The first to be identified was termed B7-1 (CD80) and shown to be a 262 amino acid polypeptide with a glycosylated molecular weight of approximately 60 kDa (Freeman et al., 1989). Subsequently B7-2 (B70/CD86) was cloned and characterised as a 323 amino acid protein glycosylated to approximately 70kDa (Azuma et al., 1993;

Freeman et al., 1993). CD86 has a slightly larger cytoplasmic domain compared to CD80, and exhibits a wider tissue distribution, being present on resting monocytes and dendritic cells, whilst CD80 expression is inducible following activation of monocytes and B cells (Azuma et al., 1993; Stack et al., 1994; Fleischer et al., 1996). Both ligands are members of the Ig superfamily and map to the same chromosomal region (3q13-23) (Fernandez-Ruiz et al., 1995) and both are capable of efficiently costimulating T cells through CD28 (Lanier et al., 1995). Recent studies have reported differences in the binding affinities of CD80 and CD86 although the functional significance of this is currently unknown (Greene et al., 1996; van der Merwe et al., 1997). The CD28 ligands have also been found on activated T cells and have been shown to be reciprocally regulated in murine T cells following activation such that CD86 expression decreases whilst CD80 is upregulated (Prabhu Das et al., 1995). Expression has also been documented on human T cell clones (Sansom and Hall, 1993; Pichler and Wyss-Coray, 1994) although little work exists on normal human T cells in this regard. The expression of costimulatory ligands on T cells themselves is intriguing, suggesting a role for T cells in the control of CD28 signalling and conferring the potential for T cell contribution to antigen presentation events.

1.2.8 CD28 and Survival

In addition to costimulating antigen-induced proliferation, CD28 signals have also been increasingly associated with promoting T cell survival and are thus of direct relevance to studies on apoptosis. Accordingly, whilst T cells from CD28 knockout mice can initiate antigen-driven proliferation, the response cannot be sustained (Lucas et al., 1995) which may be associated with decreased cell viability at late time points. Similarly, signalling through the TCR CD3 component alone can trigger apoptosis induction in *in vitro* studies, an outcome which is prevented by costimulation with anti-CD28 mAb (Boise et al., 1995; Radvanyi et al., 1996; Sperling et al., 1996) in some experimental systems. The

mechanisms which underlie CD28-mediated survival effects are not yet clear, but two potential candidates are the production of anti-apoptotic cytokines such as IL-2, and the induction of the anti-apoptotic protein $BCLX_L$ (Boise et al., 1995). Whilst the role of CD28 in the enhancement of IL-2 production is well established (Lindsten et al., 1989; Fraser et al., 1991; Fraser and Weiss, 1992; Thompson et al., 1993) the role of this cytokine in apoptosis prevention is less clear. The surprising demonstration that IL-2R knockout mice exhibit lymphoproliferation and autoimmunity as well as defects in CD95-mediated apoptosis indicates that IL-2R signalling may in fact be involved in the maintenance of self-tolerance by facilitating programmed cell death (Suzuki et al., 1995). An alternative candidate for the mediation of survival signals is the anti-apoptotic protein $BCLX_L$ which is reviewed in section 1.3.8.5. Further evidence for a role of CD28 in the promotion of cell survival is drawn from the inhibitory effect of CD28 stimulation on activation induced cell death (Groux et al., 1992; Radvanyi et al., 1996) a process of T cell elimination by apoptosis which is reviewed in section 1.3.7. However, this CD28-mediated protection remains controversial and was not observed in a number of other studies (Tan et al., 1992; Couez et al., 1994; Boehme et al., 1995).

1.2.9 CD28 Signalling

The signals responsible for mediating CD28-induced effects are not yet fully understood and are the focus of much scientific interest. Whilst the CD28 cytoplasmic tail does not posses intrinsic enzymatic activity, it contains a YMNM motif which can mediate interactions with SH2-domain containing proteins following its tyrosine phosphorylation on Y_{173} (Prasad et al., 1994; Cai et al., 1995). One such protein is the lipid/serine kinase phosphatidylinositol 3-kinase (PI3K) which has been shown to be recruited to this motif via its p85 regulatory domain following CD28 ligation (Ward et al., 1993; Pages et al., 1994). The ligand-dependent recruitment and activation of this kinase is believed to play a

key role in CD28 signal transduction as determined by mutational analysis and inhibition studies using the fungal product wortmannin to covalently bind the p110 catalytic domain of PI3K (Pages et al., 1994; Cai et al., 1995; Ward et al., 1995). Potential targets for PI3K include protein kinase B (PKB), and $p70^{S6}$ kinase (Burgering and Coffer, 1995; Downward, 1995; Franke et al., 1995). In addition, CD28 has been reported to signal via acidic sphingomyelinase generating ceramide which may mediate CD28-induced activation of c-Jun N terminal kinase (JNK) (Boucher et al., 1995). The integration and relative contribution of these pathways remains to be determined. The kinase responsible for phosphorylating the CD28 Y_{173} motif has also not yet been confirmed but one candidate is the *src* tyrosine kinase $p56^{lck}$ (Raab et al., 1995). CD28 ligation also triggers the phosphorylation of the tyrosine kinase *Itk* (August et al., 1994) however this is believed to play a negative regulatory role during T cell activation (Liao et al., 1997).

1.2.10 A CD28 homologue: CTLA4

The CD28 homologue, CTLA4 which was identified 10 years ago (Brunet et al., 1987) binds the same ligands as CD28 (CD86 and CD80) but rather than providing positive costimulatory signals, is believed to negatively regulate T cell responses (Walunas et al., 1994; Krummel and Allison, 1995; Krummel and Allison, 1996; Walunas et al., 1996). Thus the same ligands (CD80/86) can paradoxically both enhance and inhibit T cell responses depending on whether they engage CD28 or CTLA4. Whilst CD28 is constitutively expressed on the majority of human T cells (~95% of CD4+ T cells, ~50% of CD8+ T cells) (Linsley and Ledbetter, 1993; June et al., 1994), CTLA4 appears to exist primarily as an intracellular molecule and exhibits low level surface expression inducible by T cell activation (Walunas et al., 1994; Alegre et al., 1996; Wang et al., 1996a). However, in terms of ligand binding affinity, CTLA4 is markedly superior and binds with approximately 20 fold greater affinity than its homologue CD28

(Linsley et al., 1991b). Whether CD28 or CTLA4 signalling predominates in the context of ligand provision is therefore likely to depend on the balance between relative surface expression levels and relative binding affinities of these two receptors. Since CD28 and CTLA4 compete for ligand binding, interactions which downregulate CD28 expression will therefore potentially favour CTLA4 signalling. One such interaction is the binding of CD28 by its own ligands (CD80/86) which triggers rapid downregulation of CD28 messenger ribonucleic acid (mRNA) (Linsley et al., 1993). The significance of this response in terms of the effect of subsequent CD80 provision is not known.

The signals which control externalisation of CTLA4 remain to be fully elucidated although a role for Ca^{2+} signalling has been inferred (Linsley et al., 1996). Current evidence indicates that CTLA4 is retained intracellularly via an association with the AP50 component of the clathrin associated adapter AP-2 (Chuang et al., 1997; Shiratori et al., 1997). One motif implicated in this process is the CTLA4 cytoplasmic YXMN sequence which binds AP50 only when the tyrosine residue is dephosphorylated. The kinase which phosphorylates this motif, thus allowing surface expression of CTLA4, remains to be identified, as do the conditions which favour its activation. Following induction, CTLA4 appears to exhibit focal localisation towards the contact site between the T cell and APC (Linsley et al., 1996) possibly explaining why such low surface levels of this protein can be functionally significant.

A role for CTLA4 in the downregulation of T cell responses has been identified by a number of groups, however the data are presently confined to antibody-driven studies predominantly in murine systems (Walunas et al., 1994; Krummel and Allison, 1995; Krummel and Allison, 1996; Walunas et al., 1996) and an appreciation of how CLTA4 may be triggered under physiological conditions by ligand (CD80/86) engagement remains elusive. Accordingly, agonistic anti-CTLA4 antibodies have been shown to regulate T cell responses at the level of

inhibition of IL-2 production and delayed cell cycle entry (Krummel and Allison, 1996; Walunas et al., 1996). An isolated report documented the induction of apoptosis following antibody engagement of CTLA4 (Gribben et al., 1995), but this is not supported by other studies (Walunas et al., 1994; Krummel and Allison, 1996; Walunas et al., 1996). The most striking demonstration of a regulatory role for CTLA4 is the dramatic phenotype of mice deficient for this receptor which exhibit profound lymphoproliferative disease (Tivol et al., 1995; Waterhouse et al., 1995). These animals are characterised by an unchecked expansion of T cells indicative of a lack of negative regulation and this phenotype is ameliorated in the presence of CTLA4-Ig (a soluble CTLA4 construct) suggesting that the lymphoproliferation is costimulation (CD80/86) dependent (Tivol et al., 1997).

1.2.11 Relevance of CD28 and CTLA4 to T Cell Apoptosis

Since this study is an investigation of T cell apoptosis mediated by the CD95 receptor, it is of particular relevance to consider the potential effect of CD28- or CTLA4-derived signals on this pathway. In this regard, T cells which have been activated in the presence of CD28 ligation have been shown to exhibit enhanced survival (Sperling et al., 1996; Vella et al., 1997), but this has not been demonstrated to be mediated by CD95 resistance, and CD28 blockade during T cell activation triggers equivalent apoptosis in CD95-defective MRL-*lpr* mice (Noel et al., 1996b) implicating CD28 in the regulation of alternative death pathways. There is a dearth of literature which directly addresses the effect of either CD28 or CTLA4 signalling on CD95-mediated apoptosis induction and this therefore remains an important unanswered question.

1.2.12 Therapeutic Manipulation of T cell Immunity

Since effective T cell activation can orchestrate the induction of a co-ordinated immune response, the therapeutic potential for intervention at this point is

substantial. In this regard, a number of trials have demonstrated a potent immunosuppressive effect associated with blocking T cell costimulation using CTLA4-Ig to bind CD80/86. This approach has been successfully applied to transplant biology, leading to the prevention of graft rejection and the subsequent generation of tolerance (Lenschow et al., 1992; Linsley et al., 1992; Blazar et al., 1994; Pearson et al., 1994; Ibrahim et al., 1997), as well as to animal models of autoimmune disease such as murine lupus (Finck et al., 1994), murine collagen-induced arthritis (Webb et al., 1996), the multiple sclerosis model EAE (Experimental Autoimmune Encephalomyelitis) (Miller et al., 1995; Perrin et al., 1995; Racke et al., 1995; Perrin et al., 1996) and diabetes in the NOD (Non Obese Diabetic) mouse (Lenschow et al., 1995). Conversely, manipulation of the CD28/CTLA4 system can also be used to enhance immune responses, for example in the context of cancer treatment. Accordingly, antibody blocking of the CTLA4 receptor can be used to inhibit this negative regulatory pathway resulting in enhanced immunity against tumour cells (Leach et al., 1996). Despite these notable successes, unpredicted outcomes can occasionally arise during such trials including the triggering of both disease suppression and exacerbation following the administration of anti-CD80/86 antibodies to NOD mice (Lenschow et al., 1995; Lenschow et al., 1996). Such findings serve as a timely reminder of the need for further fundamental research in this area since the current working hypotheses do not adequately explain all the data. A better understanding of the events which lead to T cell activation is therefore essential both for the identification of defects underlying various diseases, and for the successful design of therapies to ameliorate these conditions.

1.3 T CELL TOLERANCE

As previously indicated, the nature of the immune system is such that a diverse array of antigen receptors is generated by gene rearrangement processes and subsequently those which may recognise self antigens and prove dangerous to the host are screened out by tolerance mechanisms. To achieve tolerance, T cells may be immunologically silenced by anergy or may be eliminated from the system by apoptosis.

1.3.1 Central Tolerance

A critical time for the generation of self-tolerance is during T cell development in the thymus which is where T cells migrate to be "educated" following their origination in the bone marrow (Kappler et al., 1987; Kappler and Marrack, 1987; Pullen et al., 1989; Jones et al., 1990). The education process involves both positive and negative selection and is based on the strength of the interaction between the TCR and the self HLA molecules which bind peptide antigens and present them to T cells. A detailed knowledge of central tolerance mechanisms is largely beyond the scope of this review but in essence T cells are required to interact sufficiently strongly with HLA molecules in order to be positively selected, but those which interact too strongly are deleted by apoptosis (negative selection) (Benoist and Mathis, 1989; Finkel et al., 1989; Ashton-Rickardt et al., 1994). The stringency of thymic selection is such that the majority of T cells generated are actually deleted at this stage of development and never enter the peripheral pool of circulating lymphocytes. Thus the generation of the T cell repertoire is a surprisingly wasteful process but this is necessary in order to achieve the fine balance between the recognition of a diverse array foreign antigens yet the avoidance of autoimmunity.

1.3.2 Peripheral Tolerance

In addition to central tolerance, peripheral tolerance mechanisms also exist to silence autoreactive cells which have eluded thymic negative selection and have escaped to the periphery (Singer and Abbas, 1994; van Parijs et al., 1996; Bluestone, 1997). The failure of thymic selection to remove all potentially autoreactive T cells is inevitable given that tissue-specific antigens are not represented in the thymus and thus may be encountered for the first time in the periphery. Similarly to central tolerance, a major mechanism of peripheral tolerance is the deletion of T cells by apoptosis, and in addition specific subsets of cells can be immunologically silenced in a process termed anergy. Interestingly, defects in central tolerance are not widely associated with the induction of autoimmunity whilst in contrast, defects in the receptors involved in peripheral tolerance mechanisms (including the death receptor CD95 and the negative regulatory receptor CTLA4) trigger the onset of a severe autoimmune phenotype (Bluestone, 1997). The appropriate induction of peripheral tolerance mechanisms is thus a key requirement for the prevention of autoimmunity under normal circumstances. However, a detailed understanding of how and when such peripheral tolerance mechanisms operate during T cell responses remains elusive.

1.3.3 CD95

Controlled cell deletion is an important mechanism of peripheral tolerance and a key receptor for this process is CD95 which triggers an apoptotic pathway when ligated. Apoptosis is a morphologically defined process of cell suicide in which a characteristic sequence of events occur including nuclear condensation, membrane blebbing and DNA fragmentation (Kerr et al., 1972). The key difference between this process and that of necrosis or "accidental" cell death is its active gene-directed nature and in fact apoptosis plays an integral role in morphogenesis during embryonic developmental as well as in tissue homeostasis (Vaux et al.,

1994). Its importance in the latter process is reflected in the derivation of its name from the Greek words "apo" meaning "from/away" and "ptosis" meaning "fall" reminiscent of the falling of the leaves in the autumn (Kerr et al., 1972).

Interest in the CD95 receptor as a transducer of an apoptotic signal began in 1989 with the development of two anti-CD95 monoclonal antibodies, APO-1 by Krammer and colleagues (Trauth et al., 1989), and anti-Fas by Yonehara and colleagues (Yonehara et al., 1989). The cDNAs for the APO-1 antigen and the Fas antigen were subsequently shown to be identical (Oehm et al., 1992). The human CD95 protein comprises 325 amino acids (306 amino acids in mouse) with a signal sequence at the N terminus and a central transmembrane domain (Nagata, 1994) indicative of a type I membrane protein. It has a molecular weight of 45kDa and is encoded by a sequence which maps to chromosome 10q24.1 in humans (chromosome 19 in mouse) (Nagata, 1994). CD95 is a member of the TNFR/NGFR family which includes the two TNFRs (type I and type II) in addition to receptors such as CD40, CD27 and CD30 (Smith et al., 1994). Northern blot analysis revealed that CD95 mRNA was expressed in a variety of tissues, and was particularly abundant in the thymus, liver, lung and kidney. Stimulation with IFNγ or TNFα triggered induction of CD95 expression in macrophages and T cells (Nagata, 1994). The CD95 receptor was shown to be a potent mediator of apoptosis by the tissue damage and lethality associated with the intraperitoneal administration of agonistic anti-CD95 antibody in mice (Ogasawara et al., 1993) and histological examination indicated severe liver damage by apoptosis in these animals. A number of further reports confirmed the role of CD95 in the transduction of an apoptotic signal, but these studies largely examined CD95 ligation in cell lines (Yonehara et al., 1989; Ogasawara et al., 1993; Suda et al., 1994; Suda and Nagata, 1994), and the role of the CD95 receptor in normal T cells was not addressed. Significantly, one early report which did encompass human peripheral blood T cells documented resistance to CD95-mediated apoptosis in activated T blasts and noted the contrast with Jurkat

T cells which exhibited sensitivity to apoptosis via this route (Miyawaki et al., 1992). Subsequently, however, two reports claimed that sensitivity of T cells to CD95-mediated apoptosis was acquired after several days of activation (Owen-Schaub et al., 1992; Klas et al., 1993).

1.3.4 CD95-Ligand

Given that CD95 had been identified as a receptor which could trigger apoptosis in susceptible cells, intense scientific interest was subsequently focused on the search for a natural ligand for this receptor. The key questions which stimulated this research were which cell type(s) express CD95L, how is this expression controlled and what is the significance of this for the induction of peripheral T cell tolerance? The ligand for CD95 (CD95L) was cloned and characterised by Suda and colleagues (Suda et al., 1994; Suda and Nagata, 1994; Takahashi et al., 1994b) by the use of a chimeric CD95-Fc protein (CD95 linked to Fc region of an immunoglobulin molecule) to enrich for CD95L-expressing cells in the cytotoxic T cell line d10S. The cloned CD95L gene was found to encode a type II membrane protein comprising 270 amino acids (Suda et al., 1994) with a molecular weight of 40kDa. A member of the TNF family, CD95L shares significant homology with other family members including TNFα, Lymphotoxin (LT) α and ß, CD40L, CD27L and CD30L (Alderson et al., 1993; Suda et al., 1994). The expression of CD95L is relatively restricted and it is predominately found on activated T (Suda et al., 1995) and Natural Killer (NK) (Arase et al., 1995) cells, although expression has also been documented on neutrophils (Liles et al., 1996), dendritic cells (Lu et al., 1997a) and macrophages (Badley et al., 1997). As for TNFα, a soluble form of CD95L can be produced following metalloproteinase cleavage (Kayagaki et al., 1995; Mariani et al., 1995) and has been detected in human serum under certain conditions (Tanaka et al., 1995). Chemical crosslinking and gel filtration analysis have indicated that human soluble CD95L exists as a trimer (Tanaka et al., 1995) consistent with the

observation that dimerisation of CD95 is insufficient to activate apoptotic signalling (Dhein et al., 1992; Kischkel et al., 1995). Interestingly, whilst murine CD95L is also proteolytically processed to a soluble form, in contrast to the situation in humans, it is unstable and non functional (Tanaka et al., 1995).

T cell CD95L expression provides one mechanism for the induction of cell death by cytotoxic T lymphocytes (CTL) (Ju et al., 1994). CTL are traditionally associated with a CD8+ phenotype, and a major mechanism underlying the induction of cytotoxicity in these cells is the production of perforin and granzymes (Jans et al., 1996; Vergelli et al., 1997). However, more recently it has been shown that CD4+ T cells can also act as CTL and that the CD95 system is particular important for cytotoxicity in this T cell subset (Rouvier et al., 1993; Hanabuchi et al., 1994; Ju et al., 1994). In this regard, it has been shown that CD4+ T cell killing of CD8+ T cells (Piazza et al., 1997), B cells (Rathmell et al., 1995) and activated macrophages (Ashany et al., 1995) can be mediated by CD95L expression.

1.3.5 Defects in CD95 or CD95L

An indication of the importance of a functional CD95 system for the maintenance of normal immune homeostasis can be drawn from the phenotype of mice which are defective in this pathway. In MRL-*lpr* mice, the CD95 gene is interrupted by an early transposable element which is inserted into intron 2 (Chu et al., 1993; Wu et al., 1993). Similarly, MRL-*lpr*^{cg} mice are characterised by a point mutation of this same allele (isoleucine replaced with asparagine in CD95 cytoplasmic region) such that whilst normal size CD95 mRNA is expressed, the receptor encoded fails to transmit an apoptotic signal (Watanabe-Fukunaga et al., 1992). These two CD95-deficient mice display analogous phenotypes to mice deficient for CD95L (MRL-*gld* mice) (Ramsdell et al., 1994b; Takahashi et al., 1994a) exhibiting lymphadenopathy from approximately 8-12 weeks of age (Sidman et al., 1992;

Gillette-Ferguson and Sidman, 1994). It is thought that the massive accumulation of T cells in the periphery of these mice is a result of defective cell removal by CD95-induced apoptosis, and importantly the onset of autoimmunity is ameliorated by T-cell specific expression of wild-type CD95 in transgenics (Wu et al., 1994), demonstrating that this phenotype is indeed attributable to the defective CD95 receptor. Thymic events do not appear to be substantially altered in these mice (Davignon et al., 1985; Herron et al., 1993; Singer and Abbas, 1994) implicating the CD95 pathway in peripheral rather than central T cell tolerance. Clearly therefore the expression of functional CD95 and CD95L is a requirement for effective immune homeostasis, however a detailed understanding of the kinetics and control of peripheral T cell elimination by CD95-mediated apoptosis is presently lacking. In particular an appreciation of which cells are eliminated and what factors control this process is lacking.

Since data from knockout mice (MRL-*lpr*, MRL-*gld*) indicate the importance of a functional CD95 pathway in the prevention of autoimmunity, the identification of similar defects in humans was of immediate interest. Children with mutations in the CD95 receptor exhibit a broadly similar phenotype to that of *lpr* mice, characterised by lymph node enlargement and autoantibody production (Rathmell and Goodnow, 1995). This syndrome has been termed Autoimmune Lymphoproliferative Syndrome (ALPS) and appears to be conferred by a dominant interfering CD95 gene mutation, possibly coding for a truncated version of CD95 (for example, lacking the death domain) which associates with normal CD95 receptors and inhibits signal transduction (Fisher et al., 1995; Cascino et al., 1996). A further two patients have been identified in whom CD95 mutations are related to an autoimmune phenotype. In these cases a 4bp insertion between exon 7 and 8 in the CD95 mRNA lead to the deletion of the cytoplasmic death domain and a consequent defect in apoptotic signal transduction (Kasahara et al., 1996).

1.3.6 CD95 and Costimulation

Interestingly, there is a small body of evidence to support a positive, possibly even costimulatory role for CD95-derived signals, in stark contrast with its more widely accepted function in apoptosis induction. Accordingly, anti-CD95 antibodies have been reported to co-operate with suboptimal TCR stimulation in the induction of T cell proliferation (Alderson et al., 1993), although these data await corroboration. In addition, T cells from CD95-defective *lpr* mice are less responsive to antigenic stimuli than are normal T cells (Davignon et al., 1985), which could be interpreted as implicating CD95 in a stimulatory role. Two potentially divergent pathways must therefore be invoked at the level of signal transduction in order for CD95 to be responsible for two such polarised outcomes, proliferation or apoptosis, and how this might be achieved under physiological circumstances is currently unknown.

1.3.7 Activation Induced Cell Death (AICD)

In addition to mediating apoptosis of target cells (Ashany et al., 1995; Rathmell et al., 1995; Piazza et al., 1997; Vergelli et al., 1997), T cell CD95L expression has also been implicated in the induction of cell suicide since T cells themselves express the CD95 receptor. A role for CD95L in cell suicide, as opposed to simply fratricide, has been confirmed by studies at the single cell level (Brunner et al., 1995) and one physiological trigger for such suicide is believed to be TCR re-engagement (Wesselborg et al., 1993). The first evidence that T cell antigen-receptor engagement might be linked to apoptosis induction came from studies on both immature (Smith et al., 1989) and mature (Groux et al., 1993) thymocytes in which anti-CD3 treatment was associated with the rapid induction of cell death. The mechanism of antigen-induced cell death was not addressed in these studies. Since double positive immature murine thymocytes express CD95 (Debatin et al., 1994) and are susceptible to apoptosis following CD95 ligation (Ogasawara et al.,

1995) a role for the CD95 pathway in antigen-induced deletion of thymocytes cannot be ruled out, and intriguingly is gaining support (Castro et al., 1996; French et al., 1997) although alternative mechanisms must compensate for this process in *lpr* mice.

The findings in thymocytes lead to similar studies using mature T cells to establish whether there was a link between antigen-receptor engagement and apoptosis induction. This work demonstrated that whilst resting T cells were relatively resistant to TCR-induced apoptosis, previously activated T cells could be triggered to undergo cell death when stimulated in this manner (Ucker et al., 1992; Wesselborg et al., 1993). In fact, sensitivity to this form of apoptosis was enhanced by cytokines such as IL-2 and IL-4 which promoted S-phase entry (Lenardo, 1991; Boehme and Lenardo, 1993). This phenomenon was termed activation induced cell death (AICD) but the mechanisms underlying this process were not known at this time.

A role for the *c-myc* proto-oncogene in AICD was inferred from the inhibition of TCR-induced apoptosis by antisense *c-myc* oligonucleotides (Shi et al., 1992) and the demonstration that the c-myc protein could directly trigger cell death under certain conditions (Evan et al., 1992). Similarly, the Nur77 family of steroid receptors have been implicated in TCR-driven apoptosis of T cell hybridomas (Liu et al., 1994; Woronicz et al., 1994) and a nuclear protein (the *TINUR* gene product) which binds the same DNA sequence as the above receptors has been shown to be induced within one hour of TCR crosslinking in a human T cell line (Okabe et al., 1995). However, the demonstration that thymic and peripheral T cell apoptosis is intact in *Nur77* knockout mice suggests a lack of involvement, or at least redundancy, in these processes under physiological conditions (Lee et al., 1995). The potential involvement of transcription factors normally associated with cellular proliferation, such as c-myc and Nur77, in cell death highlights the similarity in the signalling pathways which lead to either activation or apoptosis

and one possibility is that the coupling of these two processes may represent a safety mechanism to curb uncontrolled cell proliferation.

1.3.7.1 Role of CD95/CD95L Interactions

An exciting development in the understanding of AICD was the relatively recent demonstration that one mechanism underlying this process is the activation-driven upregulation of CD95L expression leading to cell suicide via CD95 engagement (Alderson et al., 1995; Brunner et al., 1995; Ju et al., 1995; Yang et al., 1995; Latinis et al., 1997). Thus the acquisition of cytotoxic potential via the CD95 pathway is believed to be linked to antigen-specific activation events as illustrated in figure 1.2. Further studies have defined that signalling through the ITAM (immunoreceptor tyrosine-based activation motif) of the TCR/CD3 ζ chain is sufficient for the induction of surface CD95L expression and that this event is Ca^{2+} dependent (Anel et al., 1994; Vignaux et al., 1995). In addition, protein tyrosine kinase activity (Anel et al., 1994) and macromolecular synthesis (Luciani and Golstein, 1994; Vignaux et al., 1995) are required for the induction but not the execution of CD95-based cytotoxicity following TCR ligation, in line with the need for *de novo* synthesis of CD95L. Recent work has also identified a key role for ZAP-70 (Eischen et al., 1997) and $p56^{lck}$ (Gonzalez-Garcia et al., 1997) in the TCR-driven upregulation of CD95L expression during AICD.

AICD has been particularly well documented in murine T cell hybridomas, and transformed T cell lines (Brunner et al., 1995; Ju et al., 1995; Yang et al., 1995) and it has been convincingly demonstrated that treatment of these cells with anti-CD3 antibody induces the upregulation of both CD95 and CD95L and the consequent induction of apoptosis in a proportion of the population. In addition, murine CD4+ activated T cells have been shown to exhibit high susceptibility to apoptosis following restimulation with the use of anti-CD3 (74% apoptosis) or the superantigen, Staphylococcal enterotoxin B (SEB) (68% apoptosis) (Ettinger et

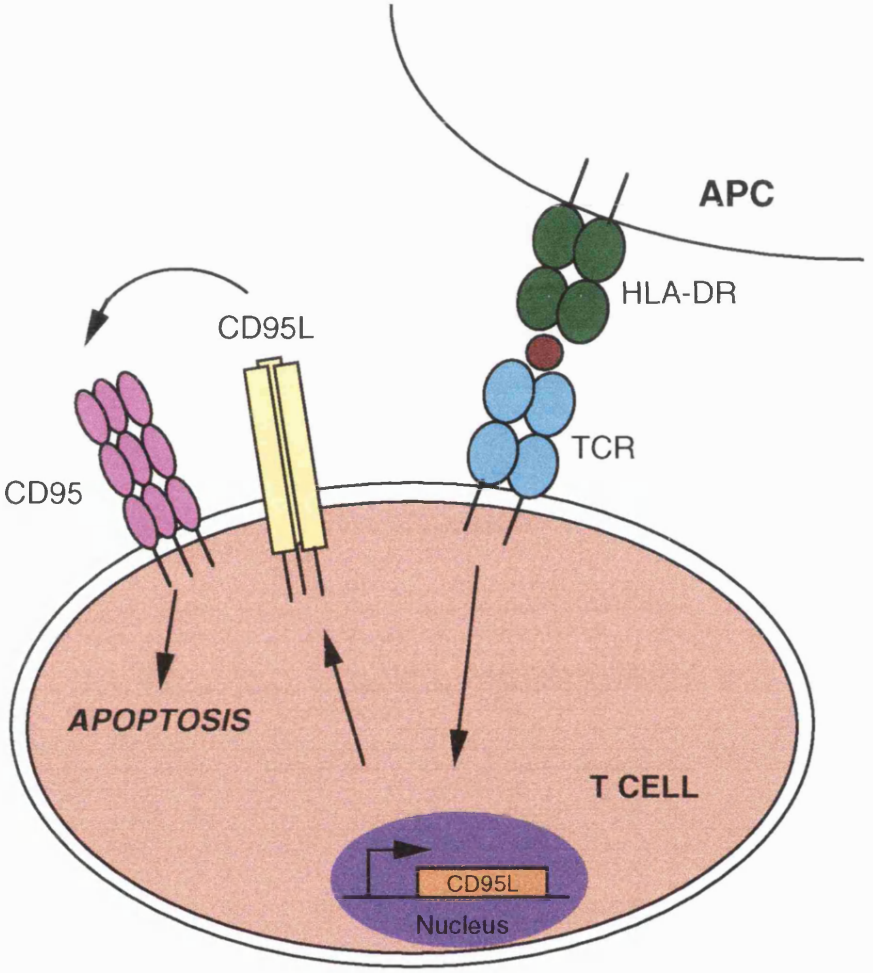


Figure 1.2: Schematic representation of activation induced cell death. Signalling through the T cell receptor results in the synthesis of CD95L which is expressed at the cell surface. The interaction of CD95L with CD95 can trigger cell suicide.

al., 1995), and furthermore this response was absent in *lpr* (CD95 defective) and *gld* (CD95L defective) mice (Zhou et al., 1992; Russell et al., 1993; Ettinger et al., 1995) indicative of a CD95-dependent mechanism. However, normal human T cells have been less extensively studied in this regard, and whilst work on human T cell clones provides clear evidence for substantial AICD (Damle et al., 1993; Alderson et al., 1995; Hargreaves et al., 1997) the existing literature on normal activated peripheral blood CD4+ T cells indicates considerable variation in the percentage of cells undergoing apoptosis in response to anti-CD3 treatment ranging from approximately thirty to fifty percent (Groux et al., 1993; Wesselborg et al., 1993; Dhein et al., 1995). The restriction of TCR-induced apoptosis to approximately thirty percent of the T cell population is in line with our own studies (Boshell et al., 1996) using superantigen-activated human peripheral blood T cells. Together, these data suggested that apoptosis was not the sole outcome of anti-CD3 treatment, at least in normal activated human T cells, and raised the question of how the choice between life and death is made within each cell under such circumstances.

1.3.7.2 Studies *in vivo*

In addition to the *in vitro* approaches outlined above, AICD has also been studied *in vivo* with the use of superantigen-mediated TCR binding. Since superantigens bypass T cell specificity by interacting with the TCR outside the antigen-binding groove, the T cells which expand under these conditions are determined by the TCR-Vß expression (Herman et al., 1991; Dohlsten et al., 1993). Accordingly, antibodies towards specific TCR-Vß chains can be used to distinguish those T cells responding to superantigen treatment providing a powerful tool for the study of immune responses. Elegant work of this nature by MacDonald and colleagues demonstrated that SEB treatment of mice triggered Vß8+ T cell expansion followed by the induction of non-responsiveness in these cells which could be attributed to a combination of anergy induction and deletion by apoptotic cell death (MacDonald et al., 1991). Similarly, injection of lymphoid cells expressing

the self superantigen Mls-1a (Minor lymphocyte-stimulating antigen-1) into mice has been shown to stimulate transient proliferation followed by anergy and partial deletion of Mls-1a-reactive T cells (Huang and Crispe, 1993). In the latter study, whilst both CD4+ and CD8+ T cells exhibited TCR downregulation, which represents one mechanism of anergy induction, apoptosis was preferentially observed in the CD4+ compartment. Negative responses such as anergy and apoptosis thus appeared to be intimately associated with antigen recognition and proliferation in these studies, implicating a role for these processes in the termination of normal immune responses. It should be noted however that certain reports have suggested that whilst T cell responses to superantigens culminate in specific T cell elimination, in contrast the stimulation of T cells with peptide leads to clonal expansion (Weber et al., 1995).

1.3.7.3 Contribution of Th1 *versus* Th2 Cells

The relative sensitivity of Th1 cells *versus* Th2 cells to AICD remains controversial. Certain reports, mainly using cloned T cell lines, maintain that CD95L is preferentially upregulated in Th1 cells leading to greater apoptosis in this subset (Ramsdell et al., 1994a; Zhang et al., 1997). One contributory factor to the increased CD95 sensitivity in Th1 cells is postulated to be the lack of FAP1 expression (Zhang et al., 1997), a phosphatase believed to negatively regulate the CD95 death pathway (Sato et al., 1995). Th2 cells, in contrast, are attributed with substantial FAP1 expression consistent with the reduced apoptotic response reported for this subset (Zhang et al., 1997). Other reports indicate no major differences between Th1 and Th2 subsets with respect to apoptosis sensitivity (Janssen et al., 1991; Russell et al., 1991; Russell et al., 1992; Watanabe et al., 1997).

1.3.7.4 Alternative Mechanisms

Importantly, despite strong evidence for a role for CD95/CD95L in AICD, it is becoming apparent that this may not be the only pathway involved in TCR-

mediated apoptosis. Accordingly, it was recently reported that although MRL-*lpr* (CD95-defective) mice were refractory to low dose anti-CD3, they exhibited massive activation induced apoptosis in response to intermediate and high doses of this antibody, indicating that a functional CD95 receptor was not absolutely required for AICD (Tucek-Szabo et al., 1996). In line with this finding, it has also been demonstrated that the TNFR offers an alternative mechanism for the mediation of AICD (Zheng et al., 1995) but that this proceeds with relatively delayed kinetics.

1.3.8 CD95 Mediated Signal Transduction

Much of the recent literature regarding CD95-mediated apoptosis pertains to the signalling mechanisms employed by this molecule and considerable progress has been made in this area. Various putative mediators have been suggested to contribute to or influence CD95-derived signalling including the phosphatase HCP (Su et al., 1995) RNA binding proteins such as TIAR (Taupin et al., 1995; Tian et al., 1995) and the tyrosine kinase *abl* (Mcgahon et al., 1995). Current ideas on the transduction of the apoptotic signal are reviewed below and a schematic diagram illustrating the main points is presented in figure 1.3.

1.3.8.1 Recruitments to CD95 Cytoplasmic Domain

CD95-mediated apoptosis can proceed in enucleated cells (Nakajima et al., 1995) and is independent of both RNA and protein synthesis (Itoh et al., 1991) suggesting that the chief components of this pathway exist in a preformed state. The CD95 cytoplasmic domain comprises 145 amino acids and lacks recognised motifs for enzymatic activity such as kinase or phosphatase activities (Itoh et al., 1991). However, a 68 amino acid shared motif between TNFR and CD95, which is necessary for transduction of death signalling, has been identified (termed the death domain) and has been shown to interact with death-domain containing proteins such as FADD (Fas associated death domain containing protein) and RIP

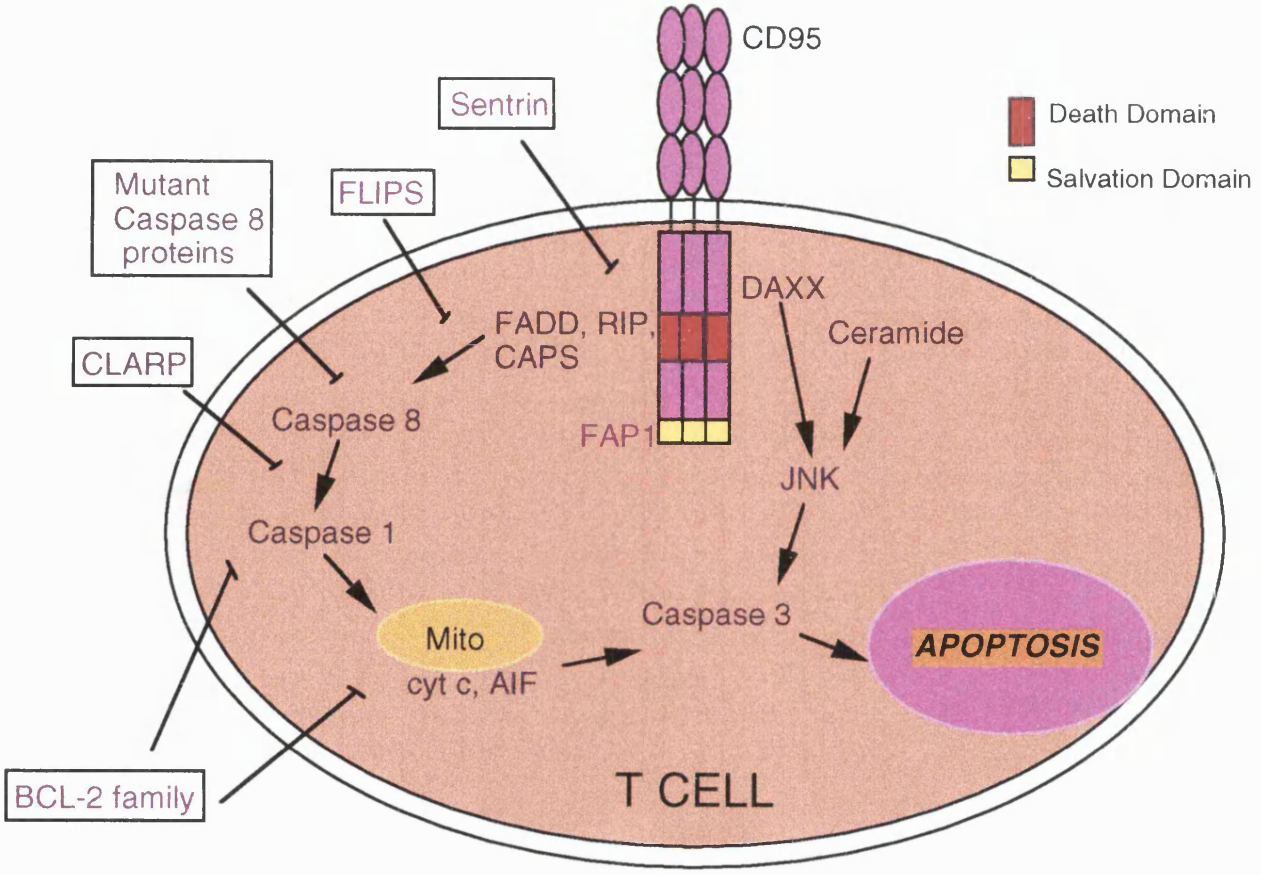


Figure 1.3: Schematic representation of the CD95 death pathway. The DISC (death inducing signalling complex) is formed by the recruitment of death domain containing proteins (such as FADD, RIP and CAPS) to the CD95 death domain. FAP1 is recruited to the CD95 salvation domain and may inhibit DISC fomation. The DISC can recruit and activate caspase 8 (FLICE). DAXX associates with CD95 cytoplasmic region and may be involved in JNK activation. The release of mitochondrial factors is thought to precede caspase 3 activation. Mito = mitochondrion, cyt c = cytochrome c, AIF = Apoptosis Inducing Factor. Arrows do not necessarily indicate a direct link.

in the yeast two hybrid system (Chinnaiyan et al., 1995; Stanger et al., 1995). Both of these proteins can induce apoptosis when overexpressed indicating a potential role in CD95 signal transduction. Moreover, the binding of FADD to the death domain appears to be dependent on the trimerisation of CD95 molecules, consistent with a triggering of this association by ligand binding (Kischkel et al., 1995) and in line with the finding that self association of the CD95 death domains is sufficient to induce a death signal (Boldin et al., 1995). Interestingly, a death domain containing protein termed reaper is present in the fruit fly *Drosophila*, indicating evolutionary conservation in the mechanism of apoptosis signal transduction (White et al., 1996). The fact that RIP contains a consensus kinase motif suggested a possible role of kinase activity in CD95-induced apoptosis, although there is little evidence to support this (Eischen et al., 1994) and the majority of studies refute this hypothesis (Schraven and Peter, 1995; Janssen et al., 1996). Further work on CD95-mediated signalling has identified the CAPS (cytotoxicity-dependent APO-1-associated proteins) which associate with oligomerised (but not monomeric) CD95: whilst CAP1 and CAP2 have been identified as serine-phosphorylated forms of FADD, CAPS 3 and 4 may represent novel apoptosis-transducing molecules (Kischkel et al., 1995).

The carboxy terminus of the CD95 receptor cytoplasmic region comprises a 15 amino acid "salvation domain" which negatively regulates CD95 signalling (Cleveland and Ihle, 1995). This region is believed to mediate the interaction with the phosphatase FAP1 (Cleveland and Ihle, 1995) and deletion of the "salvation domain" has been shown to enhance the interaction with FADD (Chinnaiyan et al., 1995). The interaction of an additional protein DAXX with the CD95 cytoplasmic domain has recently been demonstrated, and is thought to be involved in CD95-mediated JNK activation (Yang et al., 1997). The relative contribution of the signals emanating from FADD versus those derived from DAXX is incompletely understood and how these pathways may integrate to influence the final outcome of CD95 ligation remains to be elucidated.

1.3.8.2 Caspase Cascades

Over the last two years, it has emerged that a key event in the execution of the apoptotic pathway triggered by wide-ranging stimuli including CD95 is the activation of a cascade of aspartate-specific cysteine proteases (caspases). This work has been driven by studies on the nematode *Caenorhabditis elegans* which have identified a large number of genes relevant to the cell death pathway termed ced genes (*C. elegans* death genes) (Yuan and Horvitz, 1990; Hengartner, 1994; Hengartner, 1996; Henkart, 1996). Amongst the most well known are *ced-3* (for which the mammalian homologue is caspase 1) (Schwartz and Osborne, 1994; Duan et al., 1996; Kumar and Lavin, 1996; Orth et al., 1996) *ced-9* (homologous with mammalian BCL-2) (Hengartner et al., 1992; Hengartner and Horvitz, 1994) and *ced-4* (Yuan and Horvitz, 1992), for which a mammalian homologue remains to be identified (Vaux et al., 1994). Which caspases participate in the execution of apoptosis is highly cell type and stimulus dependent (Sarin et al., 1996) but CD95 killing of T cells is believed to involve sequential activation of caspase 8 (FLICE) caspase 1 (ICE) and caspase 3 (PRICE) (Los et al., 1995; Armstrong et al., 1996; Duan et al., 1996; Enari et al., 1996; Muzio et al., 1996; Anel et al., 1997). In support, caspase 1-deficient mice exhibit a defect in CD95-mediated apoptosis, yet still undergo cell death in response to dexamethasone or γ irradiation (Cleveland and Ihle, 1995). Furthermore, treatment with cell soluble fluoromethylketone caspase inhibitors such as Z-VAD-FMK (Cbz-Val-Ala-Asp(OMe)-fluoromethylketone) inhibits CD95-mediated apoptosis in numerous cell types (Sarin et al., 1996), implicating caspase activity as an essential step in the apoptotic pathway, and this inhibitor has been utilised for part of the study. Caspase 8 has been shown to interact directly with the CD95 receptor via the DISC (death inducing signalling complex), a complex formed by the recruitment of molecules such as FADD to the CD95 death domain (Muzio et al., 1996) (see figure 1.3). Caspase 8 interacts with the death effector domains (DEDs) of FADD becoming proteolytically activated as a consequence (Medema et al., 1997), and

the resistance to apoptosis in early activated T cells is reported to reflect a lack of recruitment of this caspase to the CD95 cytoplasmic domain (Peter et al., 1997).

Whilst a number of putative substrates for the caspases have been identified including β-actin, α-fodrin and lamin B1 (Lazebnik et al., 1994; Martin et al., 1995a; Martin and Green, 1995; Neamati et al., 1995) the substrate responsible for committing cells to apoptosis remains unknown. The early demonstration that one substrate for caspase 3 was the enzyme poly(ADP-ribose) polymerase (PARP) (Kaufmann et al., 1993; Lazebnik et al., 1994) suggested a possible role in the shut-down of DNA repair processes, however whilst providing a useful marker of apoptosis induction, PARP cleavage is not a requirement for apoptosis induction (Wang et al., 1997). It is of note that PKCδ represents an alternative target for caspase 3, and that overexpression of the cleavage product of this kinase is associated with the onset of characteristics of apoptosis including chromatin condensation, nuclear fragmentation and lethality both in HeLa cells and NIH3T3 cells (Emoto et al., 1995).

1.3.8.3 Role of Ceramide

The familial relationship of CD95 with the TNFR prompted signalling comparisons between these two receptors and indicated some homology, particularly in terms of the death domain and protein recruitments (Cleveland and Ihle, 1995; Boldin et al., 1996), however elements distinct to each pathway have also been identified (Schultz-Osthoff et al., 1994; Wong and Goeddel, 1994). One event which has been reported to follow ligation of CD95, TNFR and indeed CD28 is the activation of certain forms of the lipid enzyme sphingomyelinase which is responsible for mediating the cleavage of sphingomyelin to yield the signalling molecule ceramide (Boucher et al., 1995; Gulbins et al., 1995; Tepper et al., 1995; Edmead et al., 1996). Ceramide has been implicated in mediating a wide range of functions in T cells resulting in both positive and negative outcomes. Its production following CD28 ligation suggests potential involvement

in costimulatory functions (Boucher et al., 1995), whilst treatment with exogenously provided cell soluble ceramide analogues can mediate both growth arrest and apoptosis in various cell types including T cells (Jayadev et al., 1995; Tepper et al., 1995). The nature of the sphingomyelinase enzyme involved in such signalling is still subject to debate: whilst the TNF pathway utilises both acidic and neutral forms (Kolesnick and Golde, 1994; Higuchi et al., 1996), CD28 signalling involves only the acidic enzyme (Boucher et al., 1995) and there is evidence to support a role for either acidic (Cifone et al., 1993; Gulbins et al., 1995) or neutral (Tepper et al., 1995) forms in CD95-mediated signalling.

A number of putative targets for ceramide have been identified including ceramide activated protein phosphatase (CAPP) (Dobrowsky and Hannun, 1992), ceramide activated protein kinase (CAPK) (Mathias et al., 1991), JNK (Westwick et al., 1995), PKCζ (Hannun and Obeid, 1995), the proto-oncogenes *vav* and *ras* (Gulbins et al., 1995) and caspase 3 (Smyth et al., 1996) which allow ceramide to influence wide-ranging biological processes including apoptosis, inhibition of proliferation, differentiation, and inhibition of protein secretion (Hannun, 1994). Whether ceramide signals trigger positive or negative outcomes is likely to be influenced by the location and context of ceramide generation: for example ceramide-induced apoptosis in Jurkat cells is converted to a proliferative response in the presence of DAG (Hannun, 1994).

1.3.8.4 Mitochondrial Changes

Another event which has recently been implicated in apoptotic signalling is that of mitochondrial permeability transition (PT) involving a disruption in the mitochondrial inner transmembrane potential ($\Delta\Psi_m$). In viable cells, the unequal distribution of ions either side of the inner mitochondrial membrane generates both a chemical and an electrical gradient which are required for effective mitochondrial function (Attardi and Schatz, 1988). The disruption of this transmembrane potential has recently been identified as an early marker of cells

committed to apoptosis following diverse stimuli including CD95 ligation (Castedo et al., 1996) and is believed to involve a process termed permeability transition during which mitochondrial proteins can leak out into the cytosol. Such proteins include pro-apoptotic factors of which the 15kDa cytochrome c protein and AIF (apoptosis inducing factor) have been identified thus far (Liu et al., 1996; Zamzami et al., 1996). The biological properties of such factors are only just beginning to be defined, but AIF is known to proteolytically activate caspase 3 and can induce characteristic apoptotic changes when applied to isolated nuclei (Susin et al., 1997). Intriguingly, inhibition of capsase 1 prevented CD95-induced $\Delta\text{¥}_m$ disruption (Castedo et al., 1996) demonstrating that part of the caspase cascade occurs upstream of such mitochondrial changes, whilst the activation of other proteases (including caspase 3) may lie downstream of this process (Susin et al., 1997).

1.3.8.5 BCL-2 family and BCL-2-like Proteins

The cellular apoptotic response is subject to modulation by a number of intracellular proteins which either inhibit or promote cell death. The list of proteins of this nature is steadily growing and recent work has focused on the generation of hypotheses to explain their mechanisms of action, although this is still a highly controversial area. One of the first to be identified was the 26kDa BCL-2 protein which is associated with mitochondrial and perinuclear membranes (Hockenbery et al., 1990; Zehava and Cleary, 1990; Nunez et al., 1994). This protein has been shown to inhibit apoptosis induced by diverse stimuli including irradiation and glucocorticoids as well as perforin and granzymes (Chiu et al., 1995) and a reduction in BCL-2 expression correlates well with decreased T cell survival (Akbar et al., 1993a). In addition, the pathway of apoptosis induced by the transcription factor p53 is also subject to regulation by BCL-2 (Vaux et al., 1994), as is apoptosis resulting from the activation of the proto-oncogene *c-myc* (Bissonnette et al., 1992; Fanidi et al., 1992). Whilst BCL-2 was initially thought to function via an anti-oxidant mechanism (Hockenbery et al., 1993), there is now

evidence that this protein may operate by altering mitochondrial release of apoptosis-inducing factors (Shimizu et al., 1996; Kluck et al., 1997; Yang et al., 1997) and consistent with this theory, hyper-expression of BCL-2 prevents the $\Delta\Psi_m$ disruption induced by apoptotic stimuli including glucocorticoids, DNA damage, oxidants or ceramide (Zamzami et al., 1995a; Zamzami et al., 1995b). Interestingly, transfection of human or murine T cells with BCL-2 fails to block CD95-induced apoptosis, although etoposide and dexamethasone induced death are potently inhibited under these conditions (Moreno et al., 1996) suggesting that CD95 signalling is not regulated by this protein, a view supported by a number of other studies (Chiu et al., 1995; Strasser et al., 1995).

Subsequent to BCL-2 a whole family of related pro- and anti-apoptotic proteins have been identified. Pro-apoptotic intracellular proteins include BAX (21kDa) (Oltvai et al., 1993), BAK (23kDa) (Simonian et al., 1997; Ulrich et al., 1997), $BCLX_S$ (21kDa) (Boise et al., 1993) and BAD (Gajewski and Thompson, 1996; Zha et al., 1997), whilst proteins implicated in protection from apoptosis include BAG1 (Takayama et al., 1995) and $BCLX_L$ (29kDa) (Boise et al., 1993). BCL-2 and an additional anti-apoptotic protein $BCLX_L$ have been suggested to function by heterodimerisation with BAX thus preventing the formation of toxic BAX homodimers (Gajewski and Thompson, 1996). Conversely the dimerisation of a further protein BAD with BCL-2 or $BCLX_L$ serves to liberate BAX and promote apoptosis induction (Gajewski and Thompson, 1996). Recent evidence from the *C.elegans* system supports a model in which apoptosis is triggered by the release of pro-apoptotic ced-4 from its association with ced-9 (BCL-2 homologue) (Wu et al., 1997b) such that it is free to activate ced-3 (caspase 1 homologue) (Wu et al., 1997a). The relative role of the BCL-2 family members in the modulation of mitochondrial events compared to the sequestering of pro-apoptotic proteins remains to be resolved.

Intriguingly, despite their opposing functions $BCLX_S$ and $BCLX_L$ are splice variants of the same gene first identified by Boise and colleagues (Boise et al., 1993). $BCLX_L$ (the most common splice variant in both mice and humans) was found to prevent apoptosis induced by a diverse array of stimuli including glucocorticoids, γ-irradiation, Ca^{2+} ionophore and anti-CD3 antibody (Grillot et al., 1995) and has been associated with the promotion T cell survival in a number of studies (Boise et al., 1995; Gombert et al., 1996). Transfection studies in Jurkat have indicated that $BCLX_L$ expression can also prevent CD95-mediated apoptosis (Boise et al., 1995), although a similar approach in primary human and murine T cells failed to confirm this (Moreno et al., 1996). Whilst anti-CD3 treatment alone is sufficient for $BCLX_L$ induction, simultaneous CD28 ligation significantly enhances the levels of expression in a manner which inversely correlates with sensitivity to CD95-mediated apoptosis (Boise et al., 1995) indicative of a role for $BCLX_L$ in the prevention of CD95-mediated apoptosis. It is likely that the phosphorylation status and cellular localisation of such apoptosis regulatory proteins is critical in determining their function.

1.3.8.6 Negative Regulation of CD95 Signalling

Interestingly, the most recent revelations pertaining to CD95-mediated signalling have related to molecules which serve to inhibit apoptosis induction through this pathway, emphasising the stringent control mechanisms which exist to limit signalling via this receptor. Accordingly, FLICE inhibitory proteins (FLIPS) bind to the DEDs of FADD and inhibit the FADD-caspase 8 interaction (Irmler et al., 1997; Thome et al., 1997). Thus these molecules effectively act as dummy caspase 8 proteins. Two forms of the FLIP molecule appear to be produced in human leukocytes as a result of alternative splicing with the shorter form ($FLIP_S$, 28kDa) comprising two DEDs linked to a 50 amino acid carboxyterminal region and the longer form ($FLIP_L$, 55kDa) in which the two DEDs are followed by a C-terminal caspase-like domain yielding a structure reminiscent of that of caspase 8. Contrasting with caspase 8, however, $FLIP_L$ does not appear to posses protease

activity, at least not against the caspase substrates tested to date. In being recruited to the DISC in the place of caspase 8, these molecules prevent apoptotic signalling and interestingly have been found in human melanoma lesions suggesting that upregulation of FLIP may occur during tumourigenesis (Irmler et al., 1997).

Further negative regulation is made possible by the existence of mutant non-functional caspase 8 proteins FLICE-1 (Boldin et al., 1996) and FLICE-2 (Vincenz and Dixit, 1997) in which the catalytic cysteine residue has been substituted. In addition, the death effector domain-containing protein CLARP (caspase-like apoptosis regulatory protein) interacts with and regulates caspase 8 (Inohara et al., 1997) whilst the death domain binding protein sentrin can inhibit both CD95- and TNFR-induced apoptosis (Okura et al., 1996).

1.3.9 Additional Death Receptors

It has long been known that the TNF type I receptor can transmit an apoptotic signal (Wright et al., 1992; Smith et al., 1994), and in fact there is some overlap with CD95 in terms of the signalling intermediates utilised for this process (Cleveland and Ihle, 1995; Boldin et al., 1996) A recent plethora of reports have documented the existence of additional death receptors/ligands which, like CD95/CD95L, belong to the TNFR/TNF family. These include the death receptor DR-3/*wsl* which is characterised by a more restricted tissue distribution than TNFR and CD95 being predominantly expressed in spleen, thymus and peripheral blood lymphocytes (Chinnaiyan et al., 1996; Kitson et al., 1996). In addition, a cytotoxic ligand termed TRAIL (TNF-related-apoptosis-inducing-ligand) has been identified (Wiley et al., 1995) and comprises a type II membrane protein of 281 amino acids (murine version 291 amino acids) which maps to the chromosomal location 3q26. Message coding for the TRAIL molecule is found in a variety of human tissues including the spleen, lung and prostate, and in addition to the full

length membrane associated version, a soluble form of the TRAIL molecule has been detected (Wiley et al., 1995). The first receptor identified for TRAIL was designated DR4 (Pan et al., 1997) and recently two further receptors have been discovered namely DR5 and DcR1 (Sheridan et al., 1997). Intriguingly, the latter encodes a decoy receptor which inhibits TRAIL signalling and provides further evidence that apoptosis-inducing receptors are subject to tight regulation. In terms of similarity to other death-inducing ligands of this family, TRAIL exhibits closest homology to CD95L (28% identity at the C-terminal domain) closely followed by TNFα which shares 23% identity in this extracellular region (Wiley et al., 1995).

One interesting question relates to the ability of the death inducing ligands to themselves transduce signals into the cell. In this regard it is of interest that members of the TNFR family exhibit cytoplasmic domain conservation between species (although not between family members) indicating that this scenario remains a possibility. The exception to this observation is provided by TRAIL, which lacks inter-species homology in this region and is therefore unlikely itself to mediate biological functions via signal transduction (Wiley et al., 1995).

1.3.10 Medical Relevance of the CD95 Pathway

Mice injected with anti-CD95 antibody have been documented to die through liver failure with symptoms reminiscent of fulminant hepatitis in humans (Ogasawara et al., 1993). The latter is known to involve abnormally activated T cells, and in addition the transformation of hepatocytes with Hepatitis viruses B or C induces CD95 upregulation (Hiramatsu et al., 1994), indicating the potential involvement of the CD95 system in this disease. Aberrant triggering of the CD95 pathway has also been implicated in graft-versus host disease (Chu et al., 1995; Via et al., 1995; Via et al., 1996) and insulitis in IDDM (Insulin Dependent Diabetes Melitus) (Dong et al., 1995). In fact, it has recently been demonstrated that NOD mice (which represent a diabetes model) are free of insulitis when crossed with the

MRL-*lpr* (CD95-defective) mouse, illustrating a requirement for CD95-mediated cytotoxicity for the initiation of autoimmune pancreatic β cell destruction (Itoh et al., 1997).

One mechanism by which the CD95 pathway may be inappropriately triggered is if the controls on CD95L expression are compromised. It is therefore of interest that patients suffering from NK lymphoma or large granular lymphocyte leukaemia of NK or T cell type are characterised by significant serum levels of soluble CD95L, and associated systemic tissue damage, particularly in those cell types known to be most susceptible to CD95-mediated apoptosis such as hepatocytes and neutrophils (Tanaka et al., 1996). Conversely, the presence of soluble CD95 receptor may inhibit CD95-mediated apoptosis and a number of reports have suggested that this occurs in patients suffering from SLE (Systemic Lupus Erythematosus) (Poulton et al., 1995; Okubo et al., 1996; Jodo et al., 1997; Kovacs et al., 1997).

It has been proposed that apoptosis induction plays a role in the immunodepletion which characterises acquired immune deficiency syndrome (AIDS) (Groux et al., 1992; Estaquier et al., 1994; Kanagawa et al., 1995), and it has been demonstrated that T cells from Human Immunodeficiency Virus (HIV) -infected individuals are unusually susceptible to CD95-mediated apoptosis (Estaquier et al., 1995) implicating this pathway in the observed T cell depletion. Furthermore, CD95L expression has also been documented to be upregulated on HIV-infected macrophages (Badley et al., 1997) indicating a possible role in the T cell deletion which is a feature of the AIDS syndrome.

Certain tissues in the body exhibit constitutive CD95L expression, which is believed to bestow on these regions a form of immune privilege (Griffith et al., 1995; Green and Ware, 1997). These sites (including the anterior chamber of the eye comprising CD95L expressing parenchymal cells) show extended/indefinite

graft survival due to the apoptosis of infiltrating CD95 positive lymphocytes. Similar studies in *gld* mice demonstrate a lack of immune privilege implicating functional CD95L expression as a requirement for this phenomenon. Likewise, CD95L-expressing tissues are not subjected to rejection when transferred to conventional non-privileged sites: for example testis tissue survives when transplanted into renal capsule (Griffith et al., 1995). A better knowledge of how and when such death pathways operate would therefore be relevant to transplantation biology as well as to the understanding and treatment of disease states in which these controls may be lost.

1.4 AIMS OF THE STUDY

This study aimed to investigate the role of apoptosis via the death receptor CD95 in the context of human T cell activation. At the outset of this study, CD95 had been identified as a receptor that could transduce a death signal and its ligand had just been cloned. Understanding how and when CD95-mediated apoptosis operated during the course of an immune response was therefore the focus of enormous scientific interest and formed the basis of this project. In order to perform immune functions T cells require activation, thus the first aim of this study was to establish effective *in vitro* T cell activation protocols and verify that such treatments resulted in the expected proliferative and phenotypic changes. Subsequently this study aimed to establish the expression patterns of the apoptosis receptor CD95 and its ligand during the course of T cell activation, with a view to discerning the physiological circumstances under which this death pathway might be triggered. Crucially, it was hoped to gain an understanding of the factors controlling which T cells were eliminated by CD95-mediated apoptosis, and when this occurred during the course of an immune response.

Thus the aims of this project were to:-

- Establish *in vitro* T cell activation conditions which triggered effective T cell proliferation and resulted in the expected phenotypic changes.

- Determine the expression of the death receptor CD95 during the course of T cell activation.

- Investigate the sensitivity of T cells to CD95-mediated apoptosis and correlate this with the expression levels of this receptor.

- Investigate when and where CD95L was expressed and elucidate the significance of this for the regulation of T cell responses by apoptosis induction.

CHAPTER 2

Materials and Methods

2.1 MATERIALS

Sources of materials are indicated in appendix 1. All chemicals and reagents were supplied by Sigma Chemical Company Ltd. unless otherwise stated. Details of buffers and solutions can be found in appendix 3.

2.1.1 Tissue Culture Materials

Tissue culture plastics (Becton Dickinson, Falcon) were purchased from Fahrenheit Laboratory Supplies Ltd. All medium was purchased from Life Technologies and the protocols followed for medium supplementation are indicated in appendix 2. Foetal calf serum (Sigma Chemical Company Ltd) was heat inactivated. Chimeric proteins comprising the extracellular portions of CD95 or CD23 fused to the Fc region of an Ig molecule (CD95-Fc, CD23-Fc) were kindly provided by Y. Patel (Bath Institute for Rheumatic Diseases, UK) and J.-Y. Bonnefoy (Glaxo Ltd, Geneva).

2.1.2 Antibodies

Hybridomas were purchased from the American Type Culture Collection (ATCC) (Rockville, Maryland, USA) where indicated and monoclonal antibodies (mAbs) were purified in our laboratory. The control IgM antibody (Ab) OBI was a kind gift from S. Walsh (Bath Institute for Rheumatic Diseases, UK). This Ab was used as a control for the anti-Fas Ab, CH11, and is thought to bind selectively to an intracellular protein expressed by osteoblasts. FITC/PE-conjugated secondary Abs (anti-mouse polyvalent-FITC, anti-mouse IgG-FITC, anti-mouse IgG-PE) were purchased from Sigma Chemical Company Ltd. Abs utilised for intracellular cytokine detection were purchased from R&D Systems Ltd. The details pertaining to the other Abs used are presented in tabulated form on the following page.

Antigen	Clone/Antibody	Source
CD3	OKT3	ATCC
CD4	84H10	kind gift from P. Beverley, Jenner Institute, Compton, UK
CD8	UCHT4	ATCC
CD14	UCHM1	kind gift from P. Beverley
CD19	BU12	kind gift from I. MacLennan, University of Birmingham, UK
CD25	HB8784	ATCC
CD28	9.3 CD28:FITC	kind gift from P. Linsley, Bristol-Myers Squibb, Seattle, USA Serotec Ltd
CD80	BB1	kind gift from P. Linsley
CD86	IT2.2	Serotec Ltd
CD58/LFA3	TS2/9	ATCC
CD95/Fas	CH11 (IgM), ZB4 (IgG) M3 (IgG)	TCS Biologicals Ltd kind gift from D. Lynch, Immunex, Seattle, USA
HLA-DR	L243	ATCC
TCRαβ	BMA 031, FITC-conjugated	Serotec Ltd

Table 2.1: Details pertaining to antibodies utilised for the study.

2.2 METHODS

2.2.1 Tissue Culture

2.2.1.1 General Culture

Cells were cultured at 37°C and 5% CO_2 (95% air) in a humidified incubator with experimental manipulations being performed using sterile technique in a laminar flow hood (Class II). Cell lines were maintained free of mycoplasma as determined by routine testing (Mycoplasma ELISA, Boehringer Mannheim). Adherent cells were passaged by trypsinisation. Briefly, medium was removed from flasks by aspiration and cells were washed once with 10ml phosphate buffered saline (PBS, pH 7.3). Cells were incubated at 37°C for 5min in the presence of trypsin-EDTA (2ml for a 75cm^2 flask, 3 ml for a 125cm^2 flask). Trypsinisation was terminated by the addition of fresh culture medium to a final volume of 10ml and cells were passaged appropriately (routinely 1:10).

To maintain stocks, cell lines were regularly frozen for long term storage under liquid nitrogen. Cells (5 X 10^6 - 1 X 10^7) were pelleted (350g, 10min), resuspended in 20% FCS/10% DMSO and aliquoted into freezing vials. Vials were placed at -80°C for 15-18h prior to liquid nitrogen storage. Cells were thawed rapidly in a water bath (37°C), washed twice with fresh medium and returned to culture. Cell counts were performed using a haemocytometer. Unless otherwise stated, washes for all protocols were performed by the addition of PBS and cells were pelleted by centrifugation (350g) for 10min.

2.2.1.2 Transfected Cells

CHO (Chinese Hamster Ovary) cell and COS-7 cell transfectants were cultured in Dulbecco's Minimal Essential Medium (DMEM) containing 10% FCS supplemented as described (appendix 2). CHO cells stably transfected with

human cDNAs encoding the Human Leukocyte Antigen HLA-DR4, CD80 (the costimulatory ligand) or both were available in the laboratory (Sansom et al., 1993). Expression of transfected molecules was routinely checked by FACS staining. For experimental use, transfectants were routinely fixed with glutaraldehyde (0.025%, 2min) and washed twice with PBS.

2.2.1.3 Transformed T Cell Lines and T Cell Clones

Jurkat T cells (J16, ATCC) were maintained in RPMI-1640 containing 10% FCS supplemented as described (appendix 2) (complete medium).

Alloreactive human T cell clones (7418, kind gift from Eric Culbert, Zeneca pharmaceuticals, Cheshire) were maintained in RPMI-1640 supplemented with 5% human AB serum and restimulated every 14 days with glutaraldehyde-fixed B cells (ROF, ATCC) and irradiated PBMC. Briefly, ROF cells were washed once with PBS and resuspended (5 X 10^5 cells/ml) in 500µl glutaraldehyde (0.025%) for 2min then washed 3 times with medium and used at a ratio of 1:5 (ROF:7418). PBMC (1 X 10^7 cells/ml) were irradiated (3000rad) for 10min and used at a ratio of 1:1 (PBMC:7418). IL-2 (10 IU/ml) was added every 3-4 days. ROF cells were cultured in RPMI-1640 supplemented as above.

2.2.1.4 Generation of CD95-resistant Jurkat T Cells

JLW cells were generated from J16 cells by repeated challenge with the apoptotic anti-CD95 Ab CH11 (0.1µg/ml) and surviving cells were expanded in culture. Five rounds of selection were performed and the resulting population was cloned by serial dilution. The generated cell line (JLW) exhibited a significantly reduced apoptotic response to CD95 engagement compared to the parent line (J16) (figure 4.6) despite expressing equivalent levels of surface CD95. JLW cells were cultured using the same conditions as for J16 cells.

2.2.1.5 Purification of Peripheral Blood T Cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (density, 1.077 g/ml) (Boyum, 1964) of heparinsed (6 IU/ml) peripheral blood from healthy donors. PBMC were washed twice with complete medium and stimulated as indicated, or purified to T cells by immunomagnetic depletion (Sansom et al., 1993). Briefly, PMBC were incubated in complete medium (10ml) in a petri dish for 1h (37°C, 5% CO_2) to remove the adherent fraction. Non-adherent cells were recovered, washed in complete medium and incubated for 1h at 4°C with mAbs (1μg/ml) against HLA-DR, CD19 (B cell marker) and CD14 (monocyte marker) in a final volume of 500μl. Where indicated anti-CD8 mAb (1μg/ml) was also included to allow depletion of CD8+ T cells. Following washing with complete medium, cells were incubated for a further hour at 4°C with 50μl sheep anti-mouse IgG conjugated to magnetic beads (Dynabeads). Cells which had been bound by Ab could thus be removed using a magnet (negative selection by immunomagnetic depletion). FACS analysis confirmed that the resultant cell population were routinely >95% T cells as judged by positive staining with OKT3 (which recognises the CD3 component of the TCR).

2.2.1.6 Activation of Peripheral Blood T Cells

To generate activated T cell lines, PBMC were treated with the superantigen SEB (1μg/ml), or the lectins phytohaemagglutinin (PHA) (2μg/ml), or concanavalin A (ConA) (1μg/ml). Cells (1 X 10^7/ml) were cultured in complete medium or serum free medium (AIM-V) as indicated and restimulated every 10-15 days using fixed SEB-pulsed HLA-DR/CD80 transfectants (1:5 ratio transfectant: T cell).

For supernatant transfer experiments, purified T cells (1 X 10^7/ml) were stimulated using immobilised anti-CD3 and CD80 transfectants (ratio 1:5, transfectant:T cell). Anti-CD3 was plate-coated at 1μg/ml (in PBS) for 15-18h at 40μl per well in 96 well plates and wells were washed twice with PBS to remove

unbound Ab prior to the addition of cells. Following 3 days of activation in this manner, T cells were pelleted and the supernatant was applied to purified resting T cells. T cell blasts were subjected to density gradient centrifugation (as above) prior to use in apoptosis assays.

2.2.1.7 Generation of soluble CD95L

For the generation of soluble CD95L, a CD95L cDNA clone was manipulated to include a marker epitope (FLAG, Kodak) and the transmembrane and intracellular portions removed to preclude membrane binding and allow release in a soluble form. To target the resultant protein for secretion, a signal sequence (from CD80) was also added to the cDNA construct which was subsequently cloned into the eukaryotic expression vector pCDNA3 which contained a neomycin resistance gene. The vector containing this construct was then used for the transfection of COS-7 cells by electroporation. The production of the CD95L-flag construct was carried out by Dr Yusuf Patel and the transfection of COS-7 cells with this construct was performed by Dr Julie McLeod working in our laboratory. A stable line was generated by culture in the presence of neomycin (100μg/ml) to select for cells expressing the vector. Cells were then cloned into 96 well plates and supernatants were subsequently screened for apoptotic activity in the JAM assay (Matzinger, 1991) using J16 cells. Selected clones were expanded and cell supernatants were harvested, filtered and tested for specific CD95L-dependent apoptotic activity in the JAM assay.

2.2.2 Proliferation Assays

To measure the initiation of cell division in T cells subjected to various treatments, proliferation assays were employed (Waldmann et al., 1987). T cells were added to a 96 well plate (2 X 10^4 cells/well) with the treatments described in the figure legends in a final volume of 150μl of medium. Where anti-CD3 Ab

(OKT3) treatment is included, this Ab was diluted to the indicated concentration in PBS and plate-coated as described in section 2.2.1.6. Transfected cells were pulsed with superantigen (SEB, 1μg/ml) for 4h in medium (37°C, 5% CO_2) where indicated then washed three times and fixed (0.025% glutaraldehyde, 2min) prior to use in proliferation assays.

Cells were incubated at 37°C in 5% CO_2 for 3 days and pulsed with tritiated thymidine (^{3}H-thymidine) to a final concentration of 1μCi/well for the last overnight incubation. Plates were then harvested onto glass fibre filter mats (using a semi-automatic cell harvester, Skatron Ltd) and assayed by liquid scintillation counting (1290 Rackbeta, Wallac Ltd).

2.2.3 FACS staining

2.2.3.1 Surface Staining

All FACS analysis was performed on a Becton Dickinson FACStar Plus using a 100mW 488nm argon laser with light being channelled by an FL-1 filter (520nm ±20) and an FL-2 filter (580nm ±20) (Ormerod, 1990). Lysis II software (Becton Dickinson) was utilised.

To prepare T cells for FACS analysis, 1 X 10^5 cells were washed with PBS and incubated with primary staining Ab at 1μg/ml (unless otherwise stated) in a volume of 50μl for 30min at 4°C. The details of the Abs utilised for staining can be found in table 2.1 (section 2.1.2.) and for analysis of CD95 expression, ZB4 (rather than CH11) was used as the primary staining Ab. Following incubation with primary Ab, cells were washed once with PBS to remove excess Ab and then incubated for 30min at 4°C with FITC- or PE- conjugated secondary Ab. Anti-mouse polyvalent (anti-IgM, IgG, IgA)-FITC was used as the secondary Ab

unless otherwise stated and was added at a concentration of 50μg/ml in a volume of 50μl.

In certain experiments, the aim was to treat T cells with the anti-CD95 Ab (CH11) and subsequently stain for expression of surface receptors. Since CH11 is an IgM Ab, anti-mouse IgG-PE/FITC was used as a secondary Ab to allow visualisation of the staining Ab without detecting CH11. Where indicated, CHO cells were gated out during FACS analysis due to their autofluorescence in both the FL-1 and FL-2 channels following glutaraldehyde fixation.

2.2.3.2 Intracellular Staining

For intracellular cytokine staining, 5 X 10^5 T cells were stimulated for 6h with PMA (0.04μg/ml) and ionomycin (1μM) in the presence of the golgi transport inhibitor monensin (2mM) in a final volume of 500μl of complete medium. Cells were fixed and permeabilised in accordance with the "Fix and Perm" kit (TCS Biologicals) protocol and stained using FITC- or PE-conjugated antibodies (anti-IL-2-FITC, anti-IFNγ-PE, anti-IL-4-FITC) or isotype-matched controls. 10μg Ab was used in a final volume of 200μl.

2.2.4 Apoptosis Detection

2.2.4.1 Agarose Gel Analysis of Fragmented DNA

DNA fragmentation has been identified as a characteristic process during apoptosis induction (McConkey et al., 1994). To detect the presence of fragmented DNA in the cytoplasm of cells undergoing apoptosis, the following protocol was employed (Trauth et al., 1989). 1 X 10^7 Jurkat T cells were lysed on ice in 300μl of buffer comprising 10mM NaCl, 10mM Tris-HCL (pH 7.5), 1mM EDTA and 0.2% (v/v) Triton-X-100. Nuclei were pelleted by centrifugation at 15,000g for 15min at 4°C and the supernatant (containing low molecular weight

DNA) was incubated with RNAse A (final concentration 50µg/ml) for 1h at 37°C. Following addition of proteinase K (final concentration 100µg/ml) a further 4h incubation was performed at 50°C. DNA was then isolated by extracting with phenol/chloroform (5:1 v/v) twice and the aqueous phase was precipitated by addition of 0.1 X volume 3M sodium acetate and 2.5 X volume absolute ethanol for 15-18h. DNA was pelleted by centrifugation at 15,000g for 20min (4°C), washed with 70% ethanol and resuspended in 20µl Tris-EDTA. 1µl of each sample was subjected to horizontal submarine agarose gel electrophoresis using 1X TAE as a buffer. Briefly, a 2% agarose (w/v in 1X TAE) gel was made by adding 1g agarose to 50ml 1X TAE and heating in a microwave until dissolved. The liquid was allowed to cool to approximately 60°C, when ethidium bromide was added to give a final concentration of 0.5µg/ml and the gel was poured immediately. Ethidium bromide intercalates between bases within DNA or RNA and allows visualisation under ultra violet light using a transilluminator. The molecular weights of ØX174 markers (ØX174 DNA digested with *Hae* III) in base pairs are as follows: 1353, 1078, 872, 603, 310, 271, 194, 118, 72.

2.2.4.2 JAM Assay

In the JAM assay (Matzinger, 1991), cells (at a density of 1 X 10^6/ml) were pre-labelled with ^{3}H-thymidine (1.5µCi/ml) diluted in medium for 7-10h at 37°C, washed twice and subsequently cultured in fresh complete medium for a further 15-18h. Prior to experimental treatment, cells were washed once, then incubated in a 96-well plate (1 X 10^5 cells/well) in the presence of the treatments described in the figure legends at 37°C in 5% CO_2 for the indicated time periods. Treatments were performed in triplicate. Plates were harvested as described for proliferation assays (section 2.2.2.) and assayed by liquid scintillation counting. Fragmented low molecular weight DNA from apoptotic cells was not retained on the filter mat during harvesting thus apoptotic samples were distinguished by a decrease in radioactive counts. This assay was adapted for the detection of T cell cytotoxicity by co-incubation of effector T cells with ^{3}H-thymidine-labelled target

J16 cells (JAM bioassay). Target cells were labelled as described above and plated at 1 X 10^5 cells per well in a 96 well round-bottomed plate. The plate was subjected to centrifugation (60g) for 3min to pellet the cells, and the medium was removed by aspiration. Target cells were then resuspended under test conditions (i.e. in the presence of effector cells) in triplicate.

2.2.4.3 TUNEL assay

To detect fragmented DNA an *in situ* cell death detection kit (TUNEL, Boehringer Mannheim) was utilised (Negoescu et al., 1996). 5 X 10^5 cells were fixed with 100µl 4% paraformaldehyde in PBS (pH 7.4) for 30min, washed once, permeabilised with 0.1% (v/v) Triton-X-100 in 0.1% (w/v) sodium citrate (2 min, on ice) and incubated for 1h at 37°C with the reaction buffers provided in accordance with the manufacturers' instructions. Apoptosis was measured by FACS analysis as an increase in fluorescence in the FL-1 channel. For simultaneous analysis of apoptosis and surface phenotype, cells were first incubated with mAb against the surface marker of interest, stained to completion with primary and secondary Ab as described in section 2.2.3.1, following which the TUNEL assay was performed. Thus apoptosis was detected by FL-1 fluorescence and surface markers were visualised by FL-2 fluorescence.

2.2.4.4 Annexin-FITC Assay

The annexin-FITC binding assay (Apoptest^{TM}, Boehringer Mannheim) uses FITC-conjugated annexin-V to detect phosphatidylserine (PS) which is rapidly externalised during apoptosis (Martin et al., 1995b). Briefly, 3 X 10^5 cells were exposed to experimental treatment then washed once in ice cold PBS and resuspended in 100µl HEPES buffer containing annexin-FITC (2µl neat per sample) and propidium iodide (PI) (final concentration 2µg/ml). Following a 10-15min incubation at room temperature, samples were diluted 1:5 with HEPES buffer and analysed by FACS. Annexin-FITC binding was visualised as an increase in fluorescence in the FL-1 channel. This technique was adapted to allow

two colour analysis by staining for the T cell surface marker (CD58 or CD28) detected using PE-conjugated secondary Ab (as described in section 2.2.3.1) prior to resuspension in the annexin-FITC buffer, excluding the PI addition step. Accordingly, apoptosis was detected by FITC fluorescence with surface marker expression being visualised by PE fluorescence.

In certain experiments PI incorporation (5μg/ml) alone was used as a measure of cell death by allowing discrimination of cells which had lost membrane integrity and which incorporated this nuclear dye as a consequence.

2.2.5 Cell Cycle Analysis

2.2.5.1 Propidium Iodide Cell Cycle Staining

Prior to commencing the cell division process cells are referred to as existing in the G0 state and they possess one copy of DNA. When division is triggered, cells enter the G1 phase of the cell cycle and then commence the process of synthesising a second copy of their DNA during a phase termed S (synthesis) phase. When the DNA content has doubled, the cell is designated as being in the G2 phase of cell cycle which is following by the mitosis (M) phase during which the cell physically splits into two and partitions one copy of DNA into each newly formed cell. Thus cells in the G2 phase possess double the DNA content of those in the G0/1 phase whilst S phase cells possess intermediate quantities of DNA (Ormerod, 1990). Cell cycle status can be visualised by using the DNA binding dye PI to measure cell DNA content (Vindelov et al., 1983; Darzynkiewicz et al., 1992). To perform this analysis, 3 X 10^5 T cells were permeabilised in 1ml 70% ethanol (in dd$H_2$0) for 15-18h at -20°C. Cells were washed twice and resuspended in 200μl PBS containing 20μg/ml RNAse A and incubated for 30min in a cell culture incubator (37°C, 5% CO_2). PI was added to a final concentration of 100μg/ml and samples were incubated for a further 15min at room temperature.

Cells were washed once following which FACS analysis was performed. PI was visualised in the FL-2 channel using a linear scale.

2.2.5.2 Dual Staining for Cell Cycle and Apoptosis

For the simultaneous analysis of cell cycle status and apoptosis by TUNEL, the method was adapted as follows:- the ethanol incubation step was preceded by paraformaldehyde fixation (2% paraformaldehye in PBS, 15min, 4°C) and washing with 10% FCS/PBS. Following ethanol fixation (as described in section 2.3.5.1), samples were washed twice in PBS and the TUNEL reaction was performed (1h, 37°C). Samples were then washed twice in 10% FCS/0.1% (v/v) Triton-X-100 in PBS and resuspended in PI (5μg/ml) and RNAse A (200μg/ml) in a final volume of 200μl for 30min at room temperature. Samples were analysed by FACS with cell cycle being visualised in the FL-2 channel (linear fluorescence) and apoptosis being visualised in the FL-1 channel (log fluorescence).

2.2.6 Measurement of Calcium Mobilisation

Ca^{2+} mobilisation was measured using the fluo-3/acetoxymethyl ester (fluo-3/AM) -loading technique (Vandenberghe and Ceuppens, 1990). T cells were washed in Hank's balanced salt solution (HBSS) and resuspended (1 X 10^7 cells/ml) in 4μM fluo-3/AM in HBSS. Cells were incubated in the dark for 20min (37°C) then diluted 1:5 in 1% FCS/HBSS and incubated under the same conditions for a further 40min. Following washing (1% FCS/HBSS) cells were resuspended (1 X 10^6 cells/ml) in HEPES buffered saline and stored at room temperature in the dark until use. Immediately prior to analysis, cells were incubated at 37°C for 10min. Fluorescence (FL-1 channel) was measured by FACS using a linear scale. 2000 events per time point were acquired and the

presented data represent the mean fluorescence intensity (MFI) value for each time point.

2.2.7 Northern Blot Analysis

All solutions for northern blot analysis were made up in DEPC-treated water.

2.2.7.1 RNA Extraction

1 X 10^7 cells per treatment were used for RNA extraction (Chomczynski and Sacchi, 1987). Briefly, cells were dissolved in 500µl denaturing solution comprising 4M guanidinium thiocyanate, 25mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1M 2-mercaptoethanol. The following reagents were added sequentially with mixing by inversion between addition: 50µl 2M sodium acetate (pH 4.0), 500µl water saturated phenol (aquaphenol) and 100µl chloroform:isoamyl alcohol (49:1 v/v). Samples were vortexed for 10 seconds and cooled on ice for 15min followed by centrifugation at 15,000g for 10min at room temperature. The chloroform/isoamyl alcohol extraction process was repeated once more following which the aqueous (upper) layer containing RNA was precipitated with an equal volume of isopropanol at -20°C for 15-18h. The precipitate was pelleted by centrifugation (15,000g) for 10min at room temperature and redissolved in 150µl denaturing solution. Isopropanol (150µl) was used to precipitate the RNA once more in a 15-18h incubation at -20°C. Samples were centrifuged at 15,000g for 10min and pellets were washed twice with 70% ethanol and once with absolute ethanol prior to drying under vacuum for approximately 15min. The RNA pellet was resuspended in 50µl DEPC-treated water and allowed to dissolve for 15-18h at 4°C. Quantification of RNA was determined by spectrophotometry at 260nm using the equation:-

A_{260} x 40 x dilution = µg/ml RNA, where A = Absorbence

2.2.7.2 RNA Electrophoresis

For northern blot analysis, 10μg RNA was reprecipitated overnight by the addition of 1/10 volume ammonium acetate (3M) and 2 X volume absolute ethanol, and the RNA pellet was resuspended in DEPC-treated water. A 1% agarose gel was prepared by heating 4g agarose in 8ml 50X 3-[N-Morpholino]propanesulfonic acid (MOPS) and 325ml ddH_2O with 67ml formaldehyde being added once the solution had cooled to approximately 60°C. The gel was poured in a fume hood and left to set for at least 30min at room temperature. RNA was mixed with loading buffer to a volume of 50μl per sample as follows: 20μl RNA in DEPC-treated water, 20μl formamide, 7μl formaldehyde, 1μl 50X MOPS, 2μl bromophenol blue (0.025% final). Samples were heated to 70°C for 5min and chilled on ice prior to loading. The gel was run in 1X MOPS (in DEPC-treated water) at 60-80 V for approximately 6h with the buffer being continually recirculated by pumping (negative to positive).

2.2.7.3 Transfer of RNA from Gel to Membrane

After rinsing the gel with DEPC-treated water to remove formaldehyde, the blotting apparatus was set up as indicated in figure 2.2 to facilitate transfer of RNA by capillary action. The gel was placed onto a filter paper wick on a glass support in a reservoir of 20X SSC. Hybond-N membrane was cut to the same size as the gel, pre-soaked for 30min in 20X SSC at room temperature and placed directly above the gel. Several layers of absorbent filter paper were stacked above the membrane to facilitate movement of the buffer by capillary action. The assembled blotting apparatus was left for 8 -15h to allow RNA transfer.

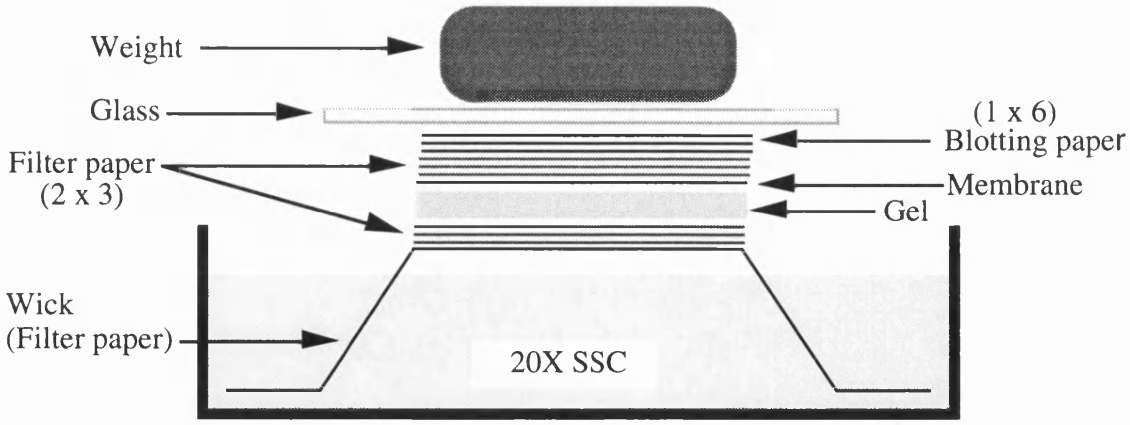


Figure 2.2: Blotting apparatus for the transfer of RNA from a formaldehyde agarose gel to a hybond membrane. The wick and stacked filter/blotting papers served to draw buffer upwards by capillary action thus facilitating transfer of RNA.

Following transfer, the gel was discarded and the membrane soaked in 50mM sodium phosphate for 10min. Subsequently, RNA was fixed by ultra violet crosslinking (2min, 1200μW/cm^2), then baking (80°C, 1H) of the membrane. Ribosomal bands were visualised by staining for 2min in methylene blue and destaining by sequential washes with DEPC-treated water. The membrane was then stored at -70°C prior to hybridisation.

2.2.7.5 Hybridisation

Full length cDNAs encoding CD58 (Seed, 1987) or CD28 (Aruffo and Seed, 1987) were used as probes for the detection of specific mRNA. Probes were labelled using a Prime-a-gene kit (Promega). Briefly, 25ng template DNA was heated to 95°C for 2min, chilled on ice and incubated for 3h at room temperature with mixed dNTP (A,T,G), nuclease-free BSA, nuclease free H_2O, Klenow, α^{32}P-dCTP and the provided buffer in a final volume of 40μl in accordance with the manufacturers' instructions. The reaction was terminated with 1.5μl of EDTA (20mM) and the percentage incorporation of the probe calculated. Accordingly, 1μl of reaction mix was applied to 2 DE81 discs: one was washed 3 times with

0.5M Na_2HPO_4 followed by ddH_2O and absolute alcohol then air dried. Both discs were counted by liquid scintillation and the percentage incorporation of radioactive counts was calculated (CPM washed disc / CPM unwashed disc X 100).

If the membrane had been previously probed, it was soaked in stripping buffer (2mM EDTA, 1% SDS) at 80°C for 30min, then rinsed with DEPC-treated water prior to re-probing. Membranes were incubated in pre-hybridisation buffer at 50°C for 2h under rotation prior to addition of the probe which was heated to 95°C immediately prior to use. Hybridisation was allowed to proceed for 15-18h at 50°C. The membrane was then washed in a prewarmed buffer (65°C) comprising 0.5% SDS in 2X SSC. A further wash with 0.5% SDS in 0.2X SSC was performed for 10min or until no background was detectable by monitoring. The membrane was then rinsed with 2X SSC and radioactivity was detected by autoradiography.

2.2.8 Data Analysis

All experiments presented were performed 3 times unless otherwise indicated in the figure legend. Data from proliferation assays and JAM assays is displayed as the mean of triplicate wells and the standard error of the mean (SEM) is shown. Statistical analysis was performed using the Mann Whitney U test where indicated and significance is taken as $p < 0.05$.

CHAPTER 3

Control of T Cell Activation

3.1 INTRODUCTION

In order to address the role of CD95-mediated apoptosis during T cell immune responses, it was first necessary to define the interactions which lead to productive T cell activation in response to antigen. An appreciation of the requirements for effective T cell stimulation, and an understanding of some of the surface receptor changes which characterise this process was considered a prerequisite for studies on the relevance of CD95 in the context of these events. In particular, it has been established that T cells are not necessarily triggered to proliferate in response to antigen engagement, but instead may become immunologically silenced (anergy) or may undergo apoptosis (Lo et al., 1988; Markmann et al., 1988; Lo et al., 1989; Ramensee et al., 1989; Schwartz, 1990). The context of antigen engagement, in terms of the additional ligands provided by the antigen-presenting cell, is a key factor in determining the nature of the response to TCR engagement, and must therefore be carefully defined prior to the study of apoptosis in activated T cell cultures. A key receptor for T cell activation is CD28 and a large body of work has established the importance of CD28 signals in costimulating antigen-mediated activation (Gimmi et al., 1991; Jenkins et al., 1991; Linsley et al., 1991a; Razi-Wolf et al., 1992; Sansom et al., 1993). Therefore initial work was carried out to establish that costimulation-dependent human T cell activation could be initiated *in vitro* and that such protocols triggered the predicted changes in T cell surface receptor expression. In addition, since a primary aim of these studies was to investigate T cell apoptosis induction, the CD28 receptor warranted particular attention as this molecule has recently been implicated in the promotion of T cell survival (Boise et al., 1995; Noel et al., 1996a; Radvanyi et al., 1996; Sperling et al., 1996). Therefore a number of experiments were carried out to investigate the expression patterns of this receptor during the T cell activation process.

T cells can be activated *in vitro* in a number of ways which fulfil the two signal requirement described earlier, including use of anti-CD3 and anti-CD28

antibodies, T cell mitogens such as PHA and ConA or superantigen. The use of superantigen is particularly suited to studies of this nature since, like peptide, it provides a TCR-derived signal and yet because it binds a site on the TCR which is distinct from the peptide-binding groove it stimulates a larger proportion of the T cell population than would specific peptide. A large proportion of this study therefore utilised T cells which were activated using the bacterial superantigen SEB and thus are referred to as SEB blasts or T blasts. These lines were generated by the addition of SEB to resting PBMC, such that T cell activation was facilitated by endogenous APCs such as monocytes. Alternatively, for experiments in which T cells were purified from the PBMC population, superantigen was pulsed onto HLA-DR expressing CHO cell transfectants which coexpressed CD80 and served as APCs to allow T cell activation. The use of CHO transfectants expressing defined combinations of T cell ligands thus provided a powerful tool for manipulating which receptors were engaged during T cell activation. Superantigen-driven T blast lines had the additional advantage that they could be readily restimulated in culture by the further addition of superantigen-pulsed HLA-DR transfectants coexpressing CD80. For the long term culture of T cells, therefore, superantigen-stimulation offered the activation model of choice, whilst for shorter term cultures a whole spectrum of activation stimuli were applicable.

3.2 RESULTS

3.2.1 An Investigation of the Requirements for T cell Activation

To control which signals were initiated during T cell activation, CHO cells expressing defined T cell ligands were utilised. These cells essentially provided a blank background for the genetic introduction of the molecule of interest and figure 3.1 illustrates the surface expression levels of these transfected ligands

(CD80, HLA-DR). The data also demonstrate that double transfectants expressed an equivalent levels of transfected molecules as single transfectants.

To test the two-signal dependence of T cell activation, proliferation assays were performed whereby resting purified T cells were treated with various combinations of stimuli and cell cycle entry was monitored by incorporation of ^{3}H-thymidine. In figure 3.2, TCR signalling was provided by immobilised anti-CD3 antibody, whilst costimulation was provided by CD80-transfected CHO cells which were fixed to preclude ^{3}H-thymidine incorporation. Clearly the presence of anti-CD3 or CD80 transfectants alone was insufficient to support productive T cell activation, whereas the provision of both signals triggered marked thymidine incorporation. This therefore established that T cells generated as the result of "two signal" protocols were activated and were proliferating.

In order to investigate variation in the temporal provision of the costimulatory signal relative to the antigen-derived signal, the addition of the CD80 transfectants was delayed following the initiation of TCR signalling using immobilised anti-CD3 antibodies. This analysis revealed (figure 3.3.A) that the addition of CD80 transfectants could be delayed for up to two hours following anti-CD3 stimulation without a detectable reduction in the proliferative response. Subsequent to this time point however, further delays in the timing of CD80 provision negatively affected T cell responses and resulted in decreased proliferation. The data also indicated that T cells which were simply retained in medium for the 12 hour delay period (i.e. in the absence of anti-CD3) did not exhibit a defect in their subsequent response to activating stimuli (column labelled CD80 + T 12h), indicating that the negative regulation was attributable to anti-CD3 signalling.

These data therefore indicated that following TCR signalling there existed a finite window of time in which a costimulatory signal could successfully be delivered via CD28. Importantly, the cells which received anti-CD3 stimulation but failed

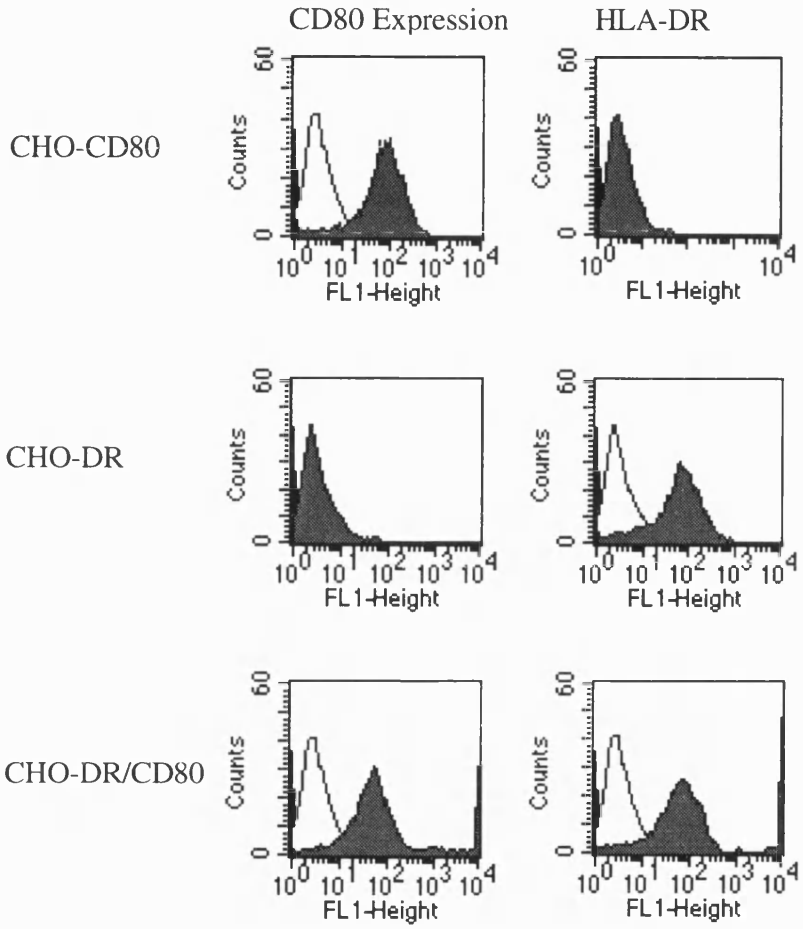


Figure 3.1: Expression of T cell ligands on transfected CHO cells. CHO cells transfected with cDNAs encoding CD80 and/or HLA-DR4 were stained for expression of CD80 and HLA-DR (filled histograms). Open histograms indicate control staining with secondary antibody alone. Surface expression of transfected cells was monitored regularly and presented data are representative of the staining routinely observed.

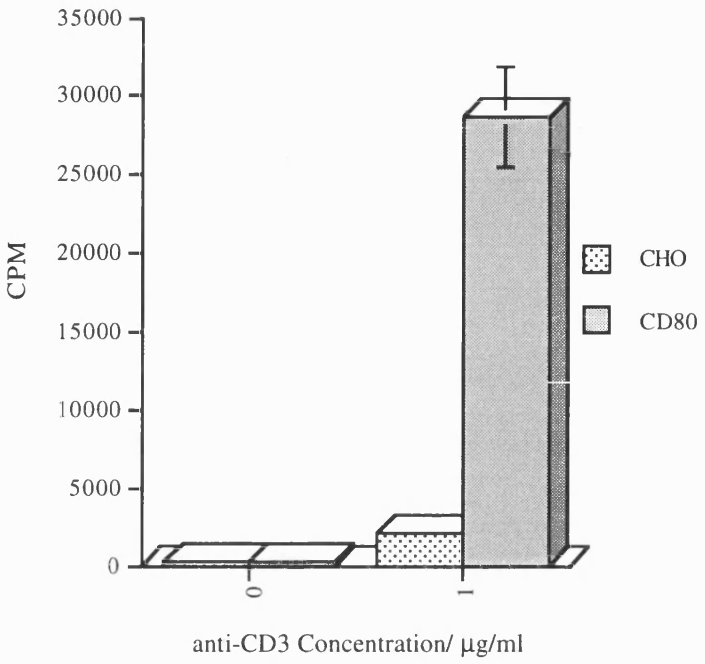
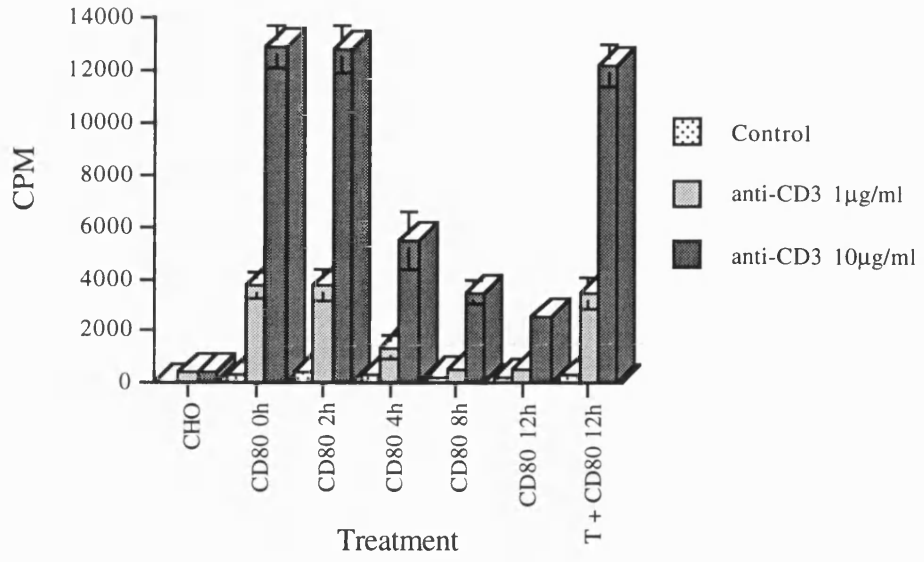


Figure 3.2: Anti-CD3 induced T cell proliferation. Purified human T cells (2 x 10^4 cells per well) were incubated with immobilised anti-CD3 (OKT3, plate-coated for 15-18h) in the presence of CD80 transfectants (ratio 5:1 T cell:transfectant) where indicated. Proliferation was assessed after 72h by incorporation of ^{3}H-thymidine and liquid scintillation counting. The mean (+/- SEM) of triplicate wells is shown. Data are representative of > 7 experiments.

(A) Proliferation



(B) TCRαβ Expression

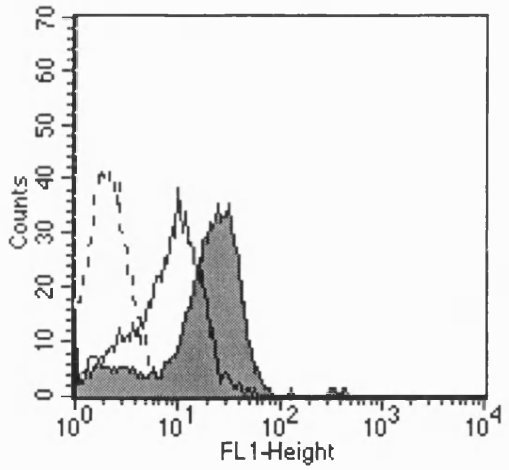


Figure 3.3: Effect of delayed addition of CD80 transfectants on anti-CD3 induced T cell proliferation. (A) Purified human T cells (2 x 10^4 cells per well) were incubated with immobilised anti-CD3 (OKT3, plate-coated for 15-18h) and CD80 transfectants (ratio 5:1 T cell:transfectant) were added after the indicated delay. Controls included the use of untransfected CHO cells and the delayed addition of both T cells and transfectants (column marked T + CD80 12h). Proliferation was assessed after 72h by ^{3}H-thymidine incorporation and the mean (+/-SEM) of triplicate wells is shown. (B) TCRαβ expression was assessed by surface staining of untreated (filled histogram) *versus* anti-CD3 (1µg/ml, 8h) treated (open histogram) T cells. Staining was carried out using a TCRαβ-FITC conjugate and the dotted line indicates staining with a control FITC conjugate. Data are representative of 3 experiments.

to proliferate due to the delay in CD80 addition nevertheless exhibited downregulation of the TCRαβ (fig 3.3.B), a phenomenon which has been identified as a characteristic mechanism of anergy induction (Huang and Crispe, 1993) and which may therefore preclude the induction of a proliferative response following subsequent antigen-presentation events.

Similarly to anti-CD3-driven proliferation, superantigen-driven T cell responses were also found to be dependent on the provision of costimulation. In figure 3.4 (panel A), resting purified T cells were stimulated with fixed transfectants (expressing HLA-DR and/or CD80) which had been previously pulsed with SEB where indicated. The resultant proliferative response was shown to be antigen-specific (SEB-dependent) and chiefly restricted to those T cells which were stimulated by transfectants expressing both HLA-DR and CD80 (figure 3.4). Interestingly, the presence of agonists for the death receptor CD95 at the time of antigen engagement always resulted in a decreased CPM relative to the maximal response although there was variability in the extent of this reduction (up to approximately 45% inhibition observed) (figure 3.4). Inhibition by anti-CD95 antibody, CH11, and soluble CD95L was comparable within each individual experiment and the inclusion of control supernatant instead of CD95L had no detectable affect. These data implied that the ligation of death receptors could potentially negatively influence the antigen presentation event, such that a diminished proliferative response ensued, and suggested that the relative provision of signalling through costimulatory receptors (such as CD28) versus death receptors (such as CD95) on T cells may be crucial in determining cell fate.

Having established successful protocols for the productive stimulation of T cells *in vitro*, the effect of this process on T cell surface receptor changes was examined. T cell activation is a complex series of events involving a number of phenotypic changes including an increase in cell size to achieve characteristic blastic morphology and alterations in the expression of various membrane

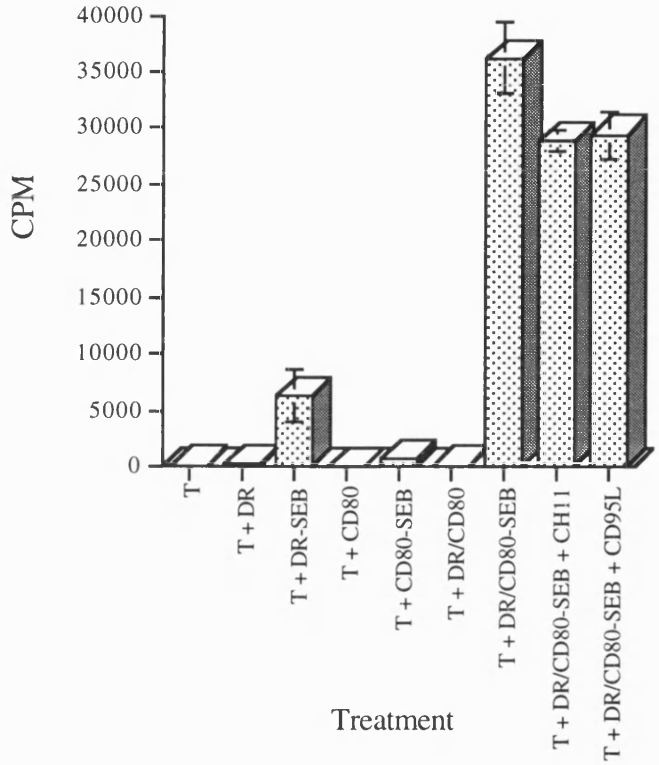


Figure 3.4 (A): SEB-induced proliferation in resting T cells. Resting human T cells (2 x 10^4 cells per well) were stimulated with fixed CHO transfectants as indicated (at a ratio of 5:1, T cell:transfectant). Transfectants were pulsed with SEB (1µg/ml, 4h) prior to fixation where indicated. CD95 engagement was provided by antibody (CH11, 0.3µg/ml) or CD95L (COS-7 cell supernatant). Column height represents the mean (+/-SEM) of triplicate wells and this experiment was repeated 5 times and similar trends were observed.

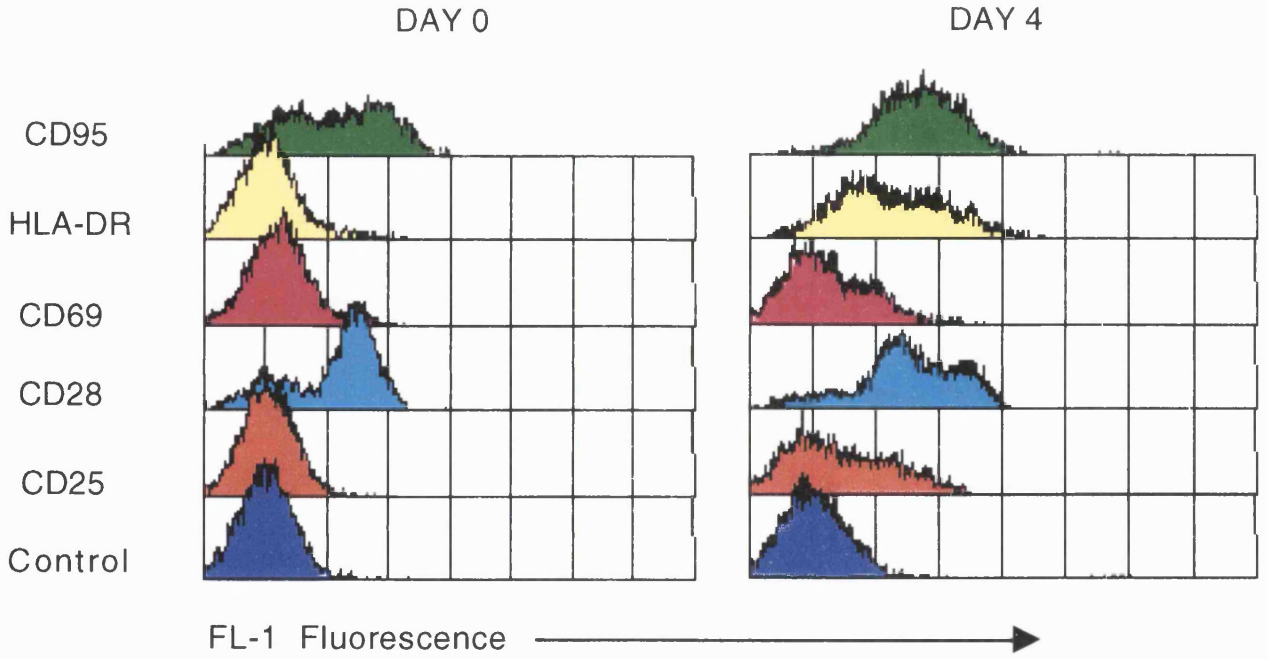


Figure 3.4 (B): SEB-induced surface marker changes in resting T cells. Resting or day 4 activated (SEB, 1µg/ml) human PBMC were stained for surface expression of CD25, CD28, CD69, HLA-DR or CD95. Control staining indicates use of secondary antibody alone. Data are respresentative of 4 experiments.

receptors. Figure 3.4 (panel B) illustrates the changing expression patterns of a selection of key T cell surface markers during the process of cellular activation, in this example mediated by superantigen stimulation. The left panel shows the constitutive expression levels of the indicated surface receptors on freshly isolated (Day 0) PBMC whilst the right panel illustrates the changes in receptor expression by day 4 following SEB stimulation. Induction of the IL-2 receptor α chain (CD25) allows the generation of a high affinity receptor for IL-2 to allow autocrine proliferation, and other markers of activation include CD69, which is thought to be involved in T cell:macrophage interactions, and HLA class II which may allow T cells themselves to take on an antigen presentation role. It was therefore possible to confirm that the activation protocols employed in these studies were competent to trigger the expected changes in T cell surface receptor expression.

In addition to altered surface receptor expression, activated T cells are also characterised by changes in functional responses to certain stimuli. Previous work in the laboratory has established a differential response to CD28 ligation in activated *versus* resting T cells, such that activated T cells can proliferate following ligation of this receptor alone whilst resting T cells require the co-ordinated provision of a TCR-derived signal (Edmead et al., 1996). To further elucidate the functional distinctions between resting and activated T cells, the response to CD3-derived signals in these cells was investigated by proliferation assay. Figure 3.5 illustrates a titration of immobilised anti-CD3 in the presence of differing ratios of CD80 transfectants:T cells. These data demonstrated that resting T cells required higher concentrations of anti-CD3 to initiate proliferation than T cell blasts, however at the highest doses of anti-CD3 (10μg/ml, 100μg/ml) there was a reduction in the proliferative response. Activated T blasts, in contrast, proliferated vigorously even at the highest concentrations of anti-CD3 indicating that activated T cells were less susceptible to negative regulation via TCR-mediated signalling than resting cells.

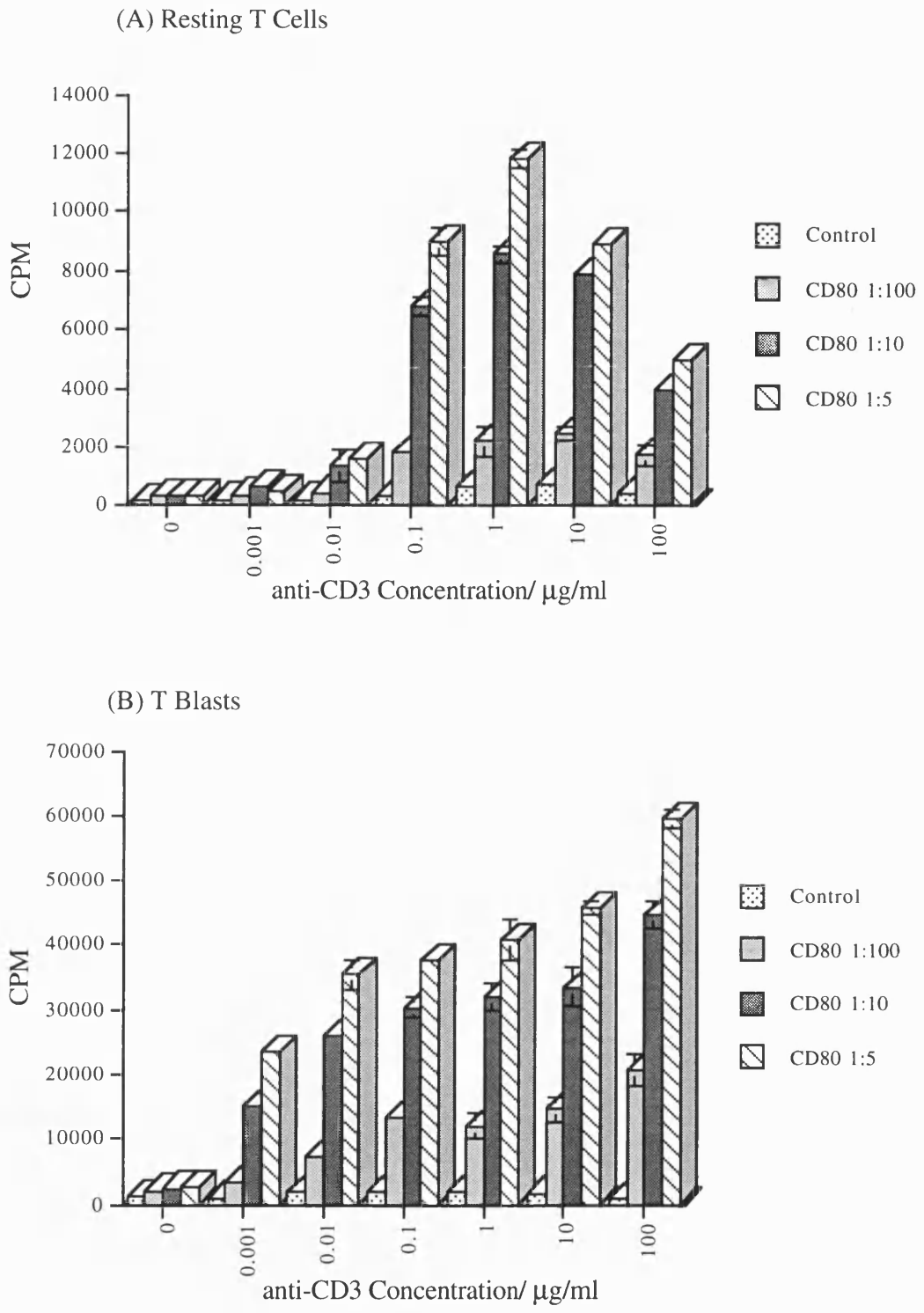
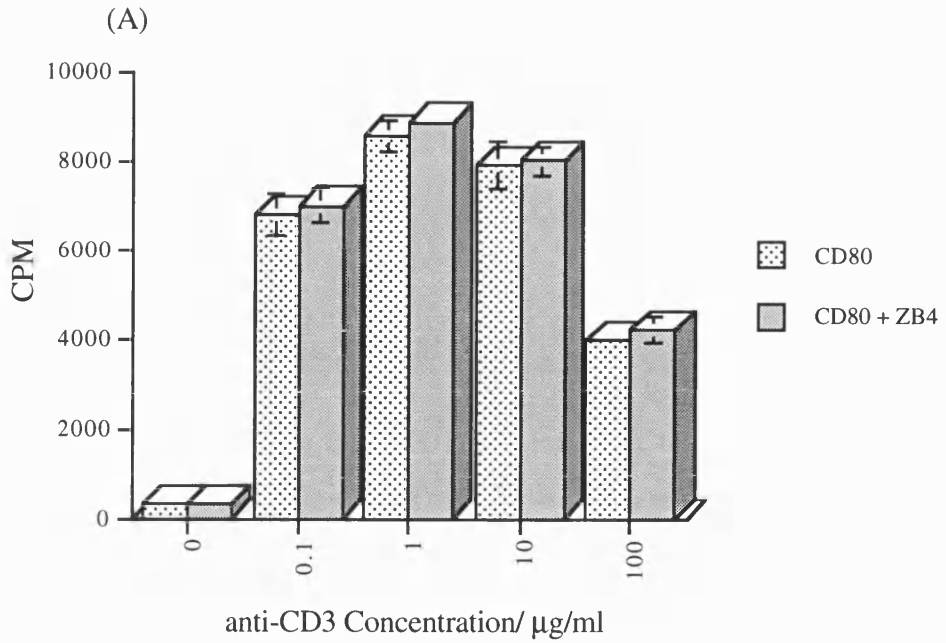


Figure 3.5. Effect of anti-CD3 titration on proliferation of resting and activated T cells. Resting T cells (A) or activated T blasts (day 12 SEB blasts) (B) were incubated with immobilised anti-CD3 in the presence of CD80 transfectants at the indicated ratios (transfectant: T cell). Proliferation was measured by ^{3}H-thymidine incorporation and the mean (+/-SEM) of triplicate wells is shown. Data are representative of 4 similar experiments.



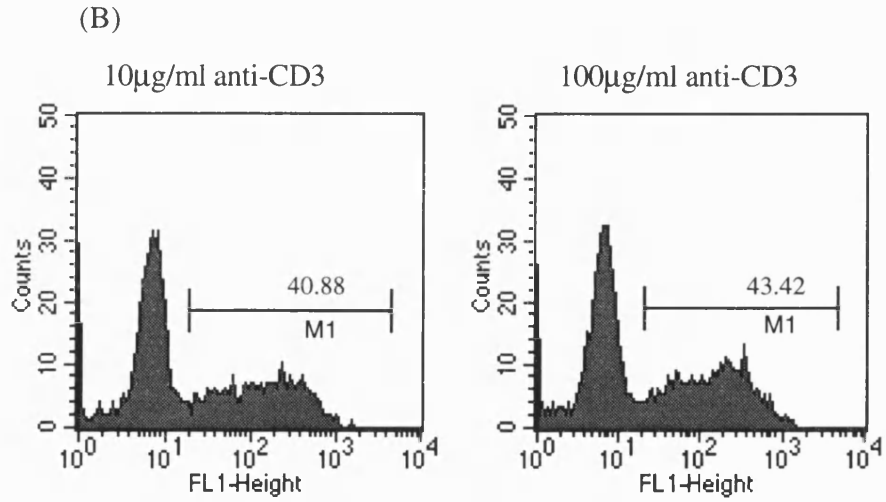


Figure 3.6: Role of apoptosis following high dose anti-CD3 treatment. (A) Purified human T cells (2 x 10^4 cells per well) were incubated for 3 days with immobilised anti-CD3 (OKT3, plate-coated at the indicated concentrations for 15-18h) in the presence of CD80 transfectants (at a ratio of 5:1, T cell:transfectant). The blocking anti-CD95 antibody ZB4 (0.5µg/ml) was included where indicated. Proliferation was assessed by incorporation of ^{3}H-thymidine and the mean (+/- SEM) of triplicate wells is shown. Data are representative of 3 experiments. (B) Samples treated with anti-CD3 (10µg/ml, 100µg/ml) in the presence of CD80 transfectants (as above) were incubated for 3 days then removed for apoptosis analysis by TUNEL. Apoptosis is visualised by increased fluorescence on the x-axis. This experiment was repeated twice with similar results.

In an attempt to elucidate the nature of the negative regulation observed in resting T cells, experiments were carried out to establish whether the reduced proliferation was a result of apoptosis induction. A major pathway for the induction of cell death in T cells is via the surface receptor CD95, therefore to preclude the triggering of this pathway, the blocking anti-CD95 antibody ZB4 was utilised. As indicated in figure 3.6 (A), the defect in proliferation at high dose anti-CD3 was not restored in the presence of ZB4 and furthermore, assessment of DNA fragmentation by TUNEL assay indicated no corresponding increase in apoptosis in these cells (figure 3.6, B), indicating that the decrease in proliferation was not attributable to the induction of apoptosis via alternative death receptors. These data indicated that the proliferative response of resting T cells was positively regulated by low concentrations of anti-CD3, but negatively regulated at high doses by a mechanism which did not involve detectable apoptosis induction, and which did not appear to be triggered in activated T cells. Further experimentation is required to establish whether this decrease in proliferation is a result of anergy induction and whether, as such, it is reversible under appropriate conditions.

3.2.2 Regulation of CD28 expression during T cell Activation

Since CD28 signals have been implicated in the promotion of cell survival, and the aims of this work were to examine the regulation of cell death, the expression patterns of this receptor during T cell activation represented an important target for investigation. It has previously been reported that engagement of CD28 by ligand (CD80/86) triggered rapid downregulation of CD28 expression both in terms of mRNA and or protein levels (Linsley et al., 1993) however the significance of this response is not known. Given that levels of CD28 expression may potentially be of relevance to T cell survival, this phenomenon was therefore studied.

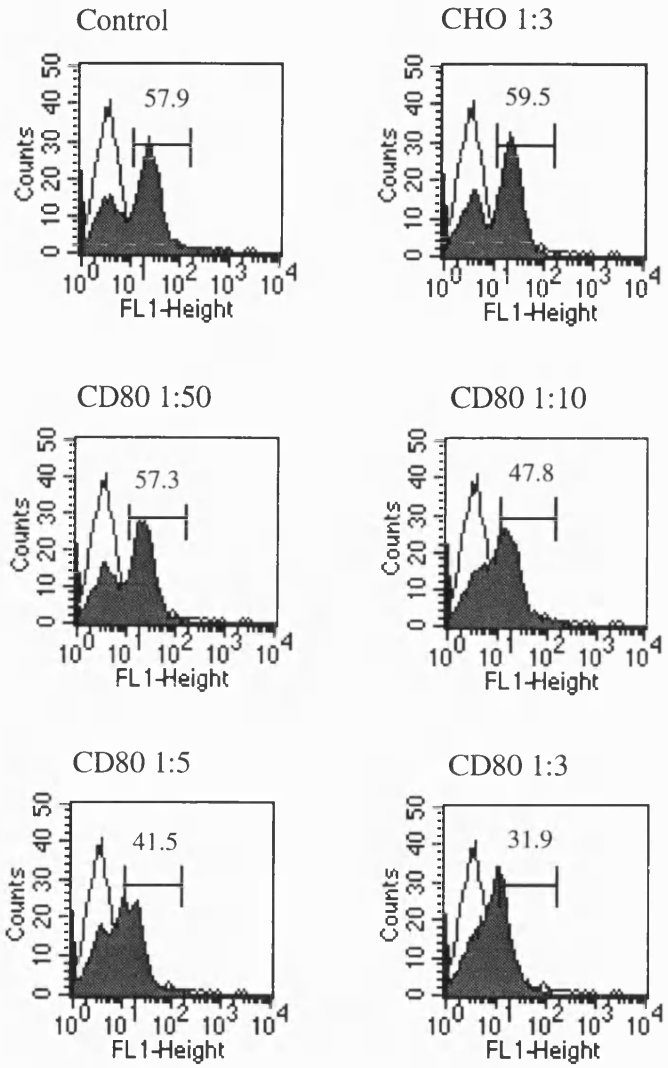


Figure 3.7: Effect of CD80 transfectants on T cell CD28 expression. Resting purified human T cells were incubated for 7h with CD80-transfected CHO cells at the indicated ratios (CHO cell:T cell) then stained for CD28 expression (filled histograms). CHO cells were gated out on autofluorescent properties during FACS analysis. Control treatment indicates the absence of transfectants and CHO treatment indicates the addition of untransfected CHO cells. Open histograms show staining with secondary antibody only. Data are representative of 4 experiments.

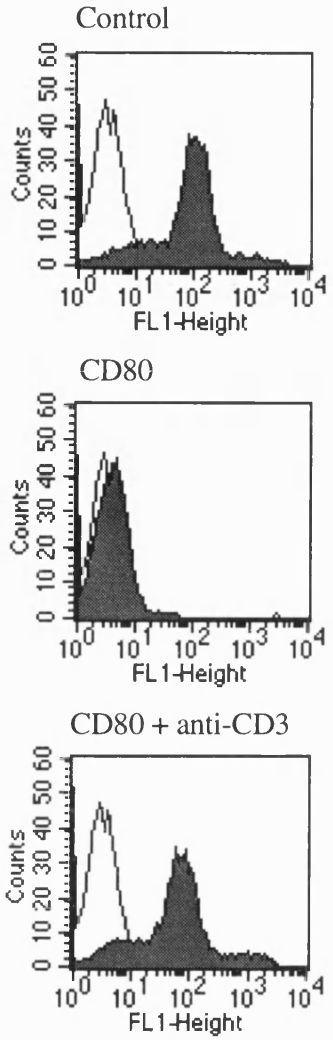


Figure 3.8: Effect of anti-CD3 treatment on CD28 re-expression following CD80-mediated CD28 downregulation. Resting T cells were treated with CD80 transfectants (at a ratio of 1:3, transfectant:T cell) in the presence of immobilised anti-CD3 (plate-coated at 1μg/ml) as indicated. After 24h cells were stained for surface expression of CD28 (filled histograms). Transfectants were gated out on autofluorescence during FACS analysis. Open histograms indicate control staining with secondary antibody alone. Data are representative of 3 experiments.

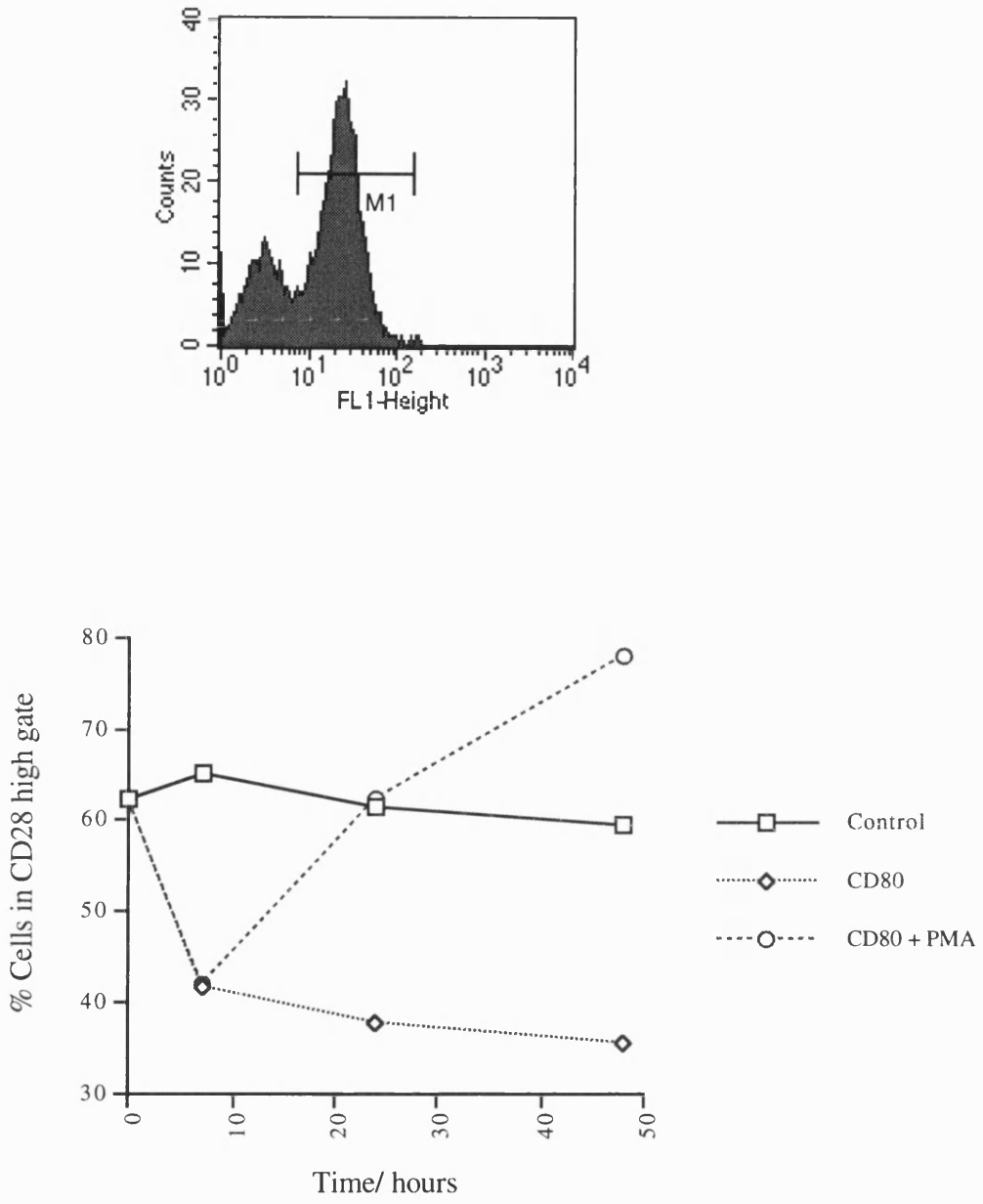


Figure 3.9: Effect of PMA on CD28 re-expression following CD80-mediated CD28 downregulation. T cells were stimulated with CD80 transfectants (at a ratio of 1:3, transfectant:T cell) in the presence or absence of PMA (0.04μg/ml) and CD28 expression measured at the indicated time points (filled histograms). The percentage of CD28 high cells is plotted according to the gate indicated in the upper panel. Data are representative of 3 experiments.

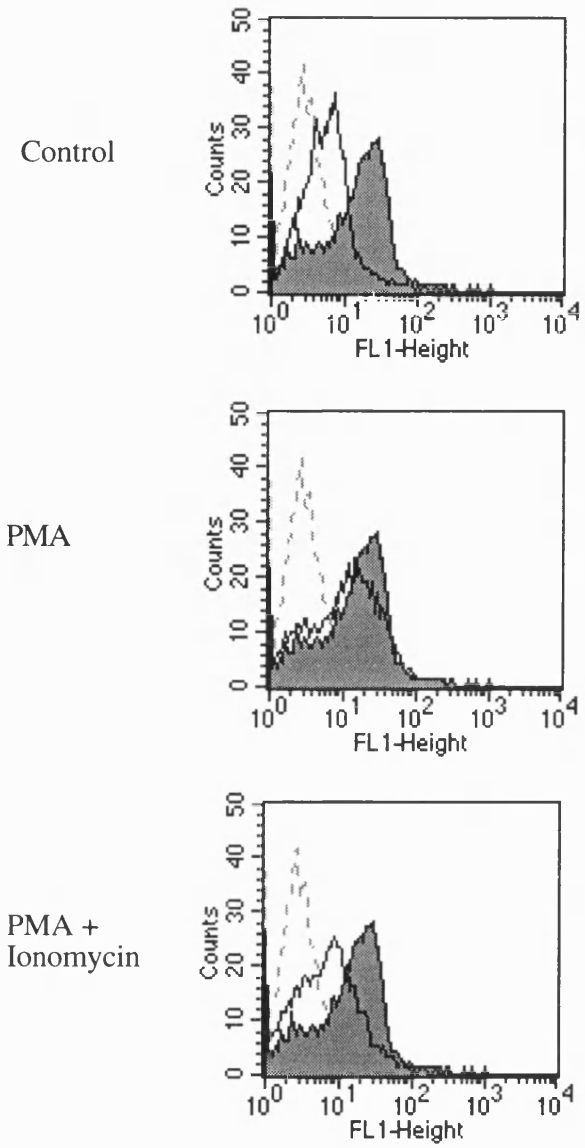


Figure 3.10: Re-expression of CD28 following treatment with CD80 transfectants in the presence of PMA and ionomycin. Resting human T cells were incubated with CD80 transfectants or untransfected CHO cells (at a ratio of 1:3, transfectant:T cell) for 48h in the presence or absence of PMA (0.04μg/ml) and ionomycin (1μM) as indicated. Cells were then stained for expression of CD28 with the CHO cells being gated out on autofluorescence during FACS analysis. The filled histogram shows CD28 staining following treatment with untransfected CHO cells alone and the same trace is plotted in each panel for comparative purposes. Solid lines represent CD28 staining following CD80 treatment. Dotted lines indicate staining with secondary antibody alone. Data are representative of 3 similar experiments.

As shown in figure 3.7, CD80-mediated CD28 downregulation was a dose-dependent phenomenon in terms of the number of CD80-transfected CHO cells present during the 7 hour incubation period, and the extent of the reduction in CD28 expression did not plateau even at high ratios of CHO-CD80 to T cells (highest ratio 1:3). In contrast, addition of untransfected CHO cells did not trigger CD28 downregulation, demonstrating that the observed decrease in CD28 expression following CHO-CD80 treatment was not due to CHO cells interfering with the staining procedure.

Data presented in figures 3.8 and 3.9 demonstrated that in order for CD28 to be re-expressed following CD80-mediated downregulation, there was a requirement for TCR engagement, or TCR-like signals i.e. PMA. In the absence of such signalling, CD28 levels remained downregulated, whereas the provision of PMA or anti-CD3 triggered rapid CD28 re-expression, ultimately reaching surface levels which exceeded those found on resting T cells (figure 3.9). Interestingly, whilst incubation with PMA was an effective trigger for CD28 re-expression, this response was weakened if ionomycin was also present (fig 3.10). Data from colleagues in the laboratory have indicated that treatment of PMA/Ionomycin (P/I) stimulated T cells with CD80 is a favourable system for the induction of CTLA4-mediated signalling (detected by a decrease in the proliferative response in the presence of CD80) and the illustration that P/I treated T cells were less effective at upregulating CD28 may be important in this regard.

3.3 DISCUSSION

The data presented in this chapter illustrate "baseline" experiments to establish that the activation conditions utilised in this study resulted in effective activation

of T cells according to a number of criteria. However they also revealed a number of interesting points. Productive initiation of T cell activation has been demonstrated to be dependent on the provision of costimulatory signals within a defined time period and in addition it has been shown that the expression of CD28, a receptor increasingly associated with cell survival, is controlled both by costimulatory and antigen-derived signals. It therefore follows that the conditions under which T cells are activated may have important consequences for their subsequent responses, including their sensitivity to apoptosis pathways such as that triggered by CD95. This observation may be of relevance in the interpretation of apoptosis studies carried out on T cells which have been "activated" in the absence of costimulatory signals (Owen-Schaub et al., 1992; Klas et al., 1993) and this will be critically analysed in more detail in chapter 6.

One consequence of antigen receptor signalling is the downregulation of the TCR (Kishimoto et al., 1995) and the data presented in figure 3.3 (B) illustrate that this response is intact even in the absence of costimulatory signals. These data indicated that a failure to receive a "second signal" within the requisite time period may potentially render T cells refractory to subsequent stimulation as a result of antigen receptor (TCR) downregulation. Such a mechanism might be important for tolerance since self antigens may be presented in the absence of costimulatory ligands whereas foreign antigens are more likely to be presented by APCs expressing upregulated CD80 levels as a consequence of pathogen-associated molecules such as lipopolysaccharide (LPS) (Razi-Wolf et al., 1992). Also apparent from these experiments is that activated T cells require a less intense TCR-derived signal to initiate proliferation compared to resting cells, in line with the findings of others (Dubey et al., 1996), and suggesting that pre-formed signalling intermediates may exist in these cells to facilitate rapid responses. A further regulatory mechanism in the control of T cell responses pertains to the nature of the antigen-derived signal, such that TCR ligation which induces high magnitude signal transduction may ultimately be a less effective trigger for T cell

activation than TCR ligation which leads to signals of an intermediate magnitude (figure 3.5). The nature of this negative regulation does not appear to involve apoptosis induction (figure 3.6), and one possibility is that high affinity TCR interactions favour CTLA4 upregulation and signalling leading to T cell silencing via this route in line with recently published findings (Alegre et al., 1996; Linsley et al., 1996). The concept that high affinity TCR ligation might trigger negative regulation in T cells is intriguing and the implications of this for self tolerance are not clear. It is possible that the nature of the interaction between antigen and TCR might represent one mechanism for the distinction between self and non-self, although other mechanisms clearly operate to influence this choice. In this regard, the rate of change of antigen dose offers one potential defining feature in the discrimination between pathogenic antigen, which is likely to rapidly increase during post-invasive replication, and host antigen which is unlikely to be subject to such rapid quantitative change.

The nature of the antigen-derived signal, as well as additional costimulatory signals, is therefore influential in determining the consequent T cell response. Likewise, the provision of ligand for death receptors may also be of relevance during the course of antigen presentation, as illustrated by the inclusion of the anti-CD95 antibody CH11 or soluble CD95L in proliferative assays. These data implied that APCs which expressed CD95L might be less effective at triggering T cell proliferation and consistent with this, it has been demonstrated that the balance between the provision of costimulatory ligand (CD80) and the provision of death-inducing ligand (CD95L) on APCs is a key determinant in the subsequent T cell response (Lu et al., 1997a; Lu et al., 1997b). It is also of note that proliferation was not entirely abrogated by the ligation of CD95 during activation (figure 3.3), indicating that whilst this pathway appeared to be activated in a subset of T cells, the universal induction of apoptosis was not triggered under these conditions. Which features distinguished the subset of T cells which were

CD95-sensitive, from those which exhibited CD95 resistance, under these circumstances thus emerged as an important question for this study.

The examination of CD28 expression revealed that this receptor was particularly tightly modulated during the T cell activation being both downregulated by CD80 binding then subsequently upregulated in a manner which was dependent on TCR or TCR-like signalling (figure 3.10). Ligand-induced downregulation of CD28 may represent a form of negative feedback to limit the extent of signalling through this receptor. In addition, and perhaps of greater significance, the downregulation of CD28 may allow the homologous molecule CTLA4 access to their shared ligands (CD80/CD86), implying that these conditions may be permissive of CTLA4-mediated signalling. In support of this hypothesis, staining data from our laboratory have indicated that CD28 and CTLA4 are reciprocally regulated during early T cell activation with surface CTLA4 expression being detectable within 4 hours of P/I stimulation. The balance between CD28-derived stimulatory signals and CTLA4-mediated negative regulation may thus depend upon the extent of CD80-mediated CD28 downregulation and the kinetics of its subsequent TCR-dependent upregulation, relative to the activation-associated induction of CTLA4 surface expression. The rapidity of CD28 re-expression would therefore be vital in determining the time window during which CTLA4 signalling could predominate, and thus could potentially dictate whether a given T cell underwent productive activation or experienced CTLA4-induced shut-down. In addition, the association of CD28 signalling with cell survival renders changes in expression levels potentially relevant to T cell sensitivity to apoptosis, for example following the ligation of receptors such as CD95, and this point is addressed in more detail in chapter 6.

A key point from these experiments is the importance of the APC in directing the T cell immune response. It has previously been established that in addition to determining which antigens are made available for T cell recognition, APCs can

also dictate whether antigen presentation results in tolerance or immunity depending on the provision of costimulation. Moreover, APCs can activate specific T cell subsets as a result of HLA class usage, manipulation of the local cytokine environment and possibly by the differential use of costimulatory ligands (Freeman et al., 1995; Kuchroo et al., 1995). Given the delicate balance between the induction of signalling through CD28 and CTLA4, it seems likely that APCs may also influence the state of T cell activation by controlling the level of ligand (CD80/86) expression, since low levels could potentially favour CTLA4 engagement. Thus APCs play a key role in determining the immunogenicity of antigens by controlling ligation of T cell "on switches", such as CD28, and T cell "off switches" such as CTLA4 and CD95.

In summary, the presented data indicated that effective costimulation-dependent T cell activation could be established *in vitro* and that the activation status of T cells could be monitored in terms of the expected changes in surface receptor expression. Productive T cell activation was shown to be dependent on the co-ordinated provision of both antigen and costimulatory signals, and in addition the timing and magnitude of signalling via these routes was shown to influence the subsequent T cell response. Staining data illustrated the dynamic regulation of T cell surface receptor expression, with particular regard to the costimulatory receptor CD28, raising the possibility that such modulation may have important consequences for the control of T cell signalling and the determination of T cell fate.

CHAPTER 4

CD95-mediated Apoptosis in T Cells

4.1 INTRODUCTION

Apoptotic cell death has emerged as a mechanism of immunoregulation and a greater understanding of how this response is initiated under physiological circumstances represents an important challenge. Unlike necrosis, apoptosis induction is an active gene-directed process such that cell surface receptors are induced for the purpose of transducing the "death signal" following ligation. Thus the coupling of a cell suicide pathway to surface receptor ligation offers a potent mechanism for the selective removal of lymphocytes during the course of an immune response or to limit T cell clonal expansion. Equally, however, the possession of such a pathway represents a potential danger to the host and the aberrant triggering of signals via this route must clearly be avoided. Receptor triggered death pathways would therefore be expected to be subject to tight controls.

One important receptor for the induction of apoptosis is CD95 which is a 45KDa transmembrane protein found on numerous cell types including T cells, B cells and monocytes (Nagata, 1994). In order to study the role of the CD95 system in T cells, experiments were performed to measure the surface expression of the CD95 receptor on T cells during the process of cellular activation, and to elucidate the ability of this receptor to transmit an apoptotic signal following ligation. As a pre-requisite to this investigation, a number of assays to measure apoptosis were established and in addition a CD95-insensitive cell line and a source of soluble CD95L were generated.

4.2 RESULTS

4.2.1 Establishment of Apoptosis Assays

Apoptotic cell death is distinguished from necrosis on the basis of morphological criteria which include chromatin condensation, membrane blebbing and the partitioning of fragmented DNA into membrane-bound apoptotic bodies (Kerr et al., 1972; McConkey et al., 1994). A characteristic feature of apoptosis is the systematic cleavage of nucleosomal DNA into first high molecular weight (300kbp and 50kbp) then low molecular weight (oligomers of 180bp) fragments (McConkey et al., 1994). One of the earliest methods of detecting whether cells were undergoing apoptosis was therefore to extract the cellular DNA and run it on an agarose gel such that fragmented DNA could be visualised as laddering. In order to establish assays to measure apoptosis induction, DNA laddering was directly compared with a PI exclusion assay in which cell death was measured by nuclear staining following the loss of plasma membrane integrity. Thus, cells in the late phases of apoptosis fail to exclude the DNA-binding dye PI and the resultant red fluorescence can be monitored by FACS at the single cell level.

To induce apoptosis, the anti-CD95 antibody CH11 was utilised in the presence or absence of a blocking anti-CD95 antibody (ZB4) or an inhibitor of CD95 signal transduction (Z-VAD-FMK) (Sarin et al., 1996). Jurkat T cells (J16) were treated for 15 hours with the indicated reagents following which samples from the same incubation were assayed using the two detection methods indicated (figure 4.1). The data generated from the PI exclusion assay (figure 4.1, A) indicated that, in this experiment, CH11 treatment induced a 62% increase in the PI bright (non viable) population and additionally the cell shrinkage associated with apoptosis could be visualised as a decrease in forward light scatter (FSC). Consistent with this picture, a smear which is characteristic of degraded DNA was clearly visible in the CH11-treated sample, but absent from the control lane (figure 4.1, B).

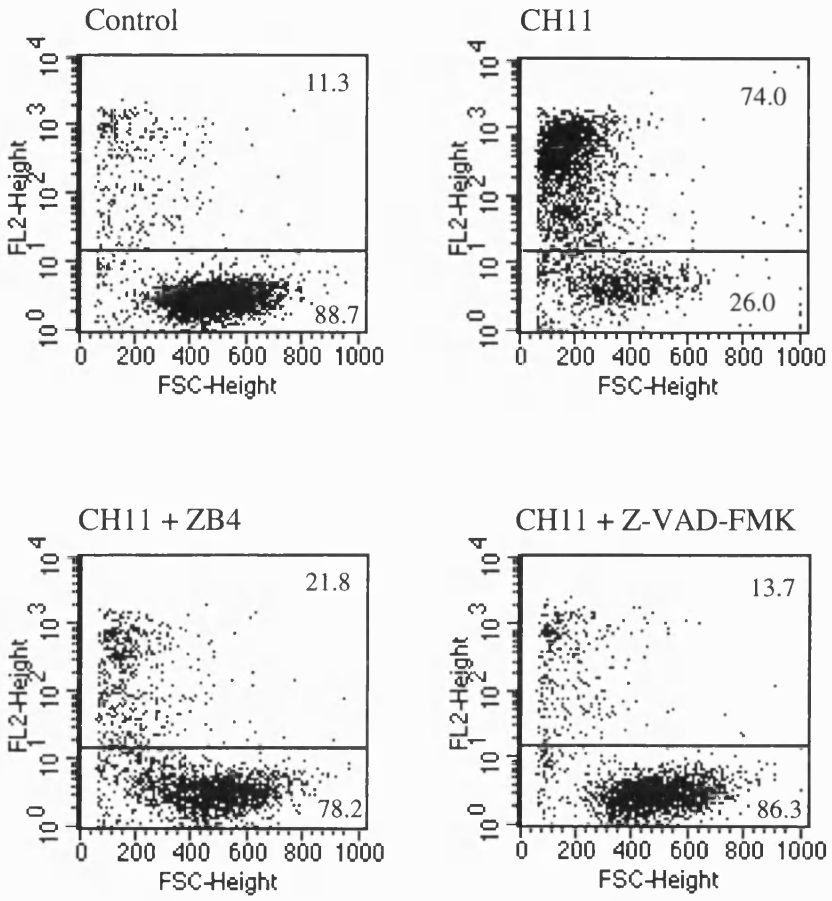


Figure 4.1 (A): Apoptosis measurement by propidium iodide (PI) staining. Jurkat T cells (5 x 10^5 cells per treatment) were incubated for 15h with medium (control), the apoptotic anti-CD95 antibody CH11 (0.05µg/ml), the blocking anti-CD95 antibody ZB4 (0.5µg/ml) or the caspase inhibitor Z-VAD-FMK (10µM) as indicated. 10^5 cells were removed for PI (5µg/ml) staining whilst the remainder were reserved for DNA laddering analysis (figure 4.1, B) These data are representative of 2 experiments.

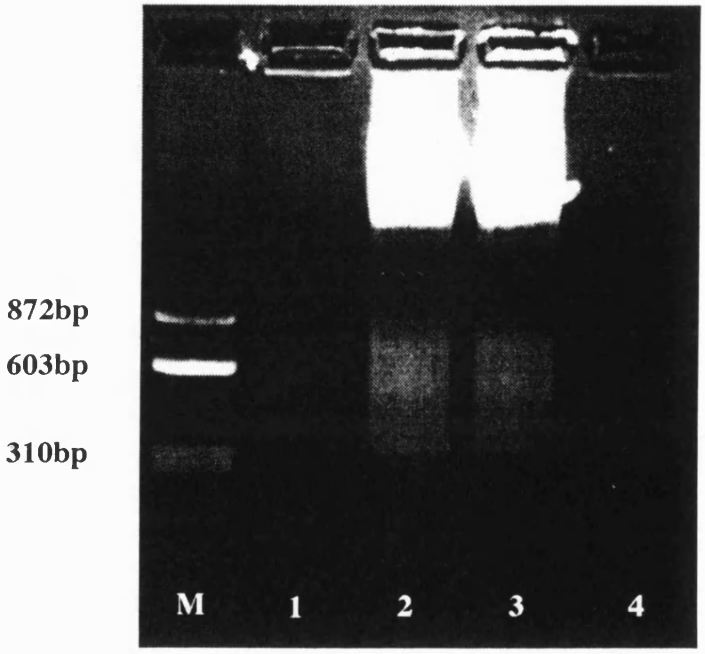


Figure 4.1 (B): Apoptosis measurement by DNA laddering analysis. Jurkat T cells (5 x 10^5 cells per treatment) were incubated for 15h with medium, the apoptotic anti-CD95 antibody CH11 (0.05µg/ml), the blocking anti-CD95 antibody ZB4 (0.5µg/ml) or the caspase inhibitor Z-VAD-FMK (10µM) as indicated. Cells were lysed and analysed for DNA laddering by agarose gel eletrophoresis as described in the methods section. Lane 1= medium, Lane 2 = CH11, Lane 3 = CH11 + ZB4, Lane 4 = CH11 + Z-VAD-FMK. Data are representative of 2 experiments.

Since this protocol involved the initial removal of whole nuclei, only low molecular weight fragmented DNA remained for analysis, explaining the lack of material in non-apoptotic lanes. Since apoptotic cells have undergone DNA fragmentation, even after the nuclei have been pelleted, low molecular weight DNA still remains in the cytoplasmic fraction, and can be isolated using this protocol. The apoptotic response was almost completely inhibited in the presence of the caspase inhibitor Z-VAD-FMK as measured by PI exclusion, and in line with this result there was no detectable DNA laddering in this sample. However, cells which were treated with CH11 in the presence of the blocking anti-CD95 antibody ZB4 were not entirely spared from apoptosis and exhibited a small (10%) increase in the proportion of PI bright (non viable) cells in this experiment. Interestingly, the DNA laddering assay failed to distinguish the sample in which there was 10% apoptosis from that in which there was 62% apoptosis, since there was comparable DNA smearing in both lanes. One possibility is that the difference between treatments is masked by gel overloading although electrophoresis of decreased amounts of sample did not support this hypothesis. Repeated experiments revealed a similar trend, indicating a potential limitation associated with the use of DNA laddering assays for the quantitative measurement of apoptosis in that even low levels of apoptosis resulted in marked DNA ladders raising the concern that the degree of apoptosis within a cell sample could be overestimated by this method. Equally, the protective effect of certain treatments (such as the blocking anti-CD95 antibody, ZB4) could be missed if the visualisation of DNA laddering on agarose gels were to be relied upon as a measure of apoptosis.

Since the degree of DNA laddering did not appear to provide an adequate reflection of the proportion of apoptotic cells within a sample, alternative assays were investigated with a view to providing a more quantitative approach to apoptosis detection. The JAM assay (Matzinger, 1991) allowed apoptosis to be detected as a measure of DNA fragmentation by pre-labelling cells with ^{3}H-

thymidine and harvesting through a glass fibre filter mat following experimental treatment. Fragmented DNA from apoptotic samples is not retained on the mat and apoptosis can therefore be detected by a decrease in radioactive counts. In order to set up this assay for routine use in the laboratory, a number of preliminary investigations were carried out to establish a suitable cell labelling protocol in addition to appropriate cell numbers and cell incubation times. As demonstrated in figure 4.2, the resultant assay provided a sensitive and quantitative measure of apoptosis induction since the anti-CD95 antibody decreased radioactive counts (CPM) in a dose-dependent (A), time-dependent (B) and ZB4 blockable (C) manner.

During the course of these studies, two further apoptosis assays became available for use, namely the TUNEL assay (Negoescu et al., 1996) and the annexin-FITC binding assay (Martin et al., 1995b). The former used DNA end-labelling with fluorescence-tagged nucleotides to allow cells with fragmented DNA to be distinguished by increased fluorescence. This method required that cells were fixed and permeabilised following experimental treatment, and could only detect apoptosis once the DNA cleavage stage had been reached. The annexin-FITC assay, in contrast, allowed identification of apoptotic cells at very early time points since it was based on changes in the phospholipid bilayer which occurred shortly after the triggering of an apoptotic stimulus (Martin et al., 1995b). FITC-conjugated annexin-V was used to bind PS, a lipid normally restricted to the inner leaflet of the plasma membrane, but which is actively externalised as a rapid response to apoptotic signalling (Martin et al., 1995b). In certain cell types, though not in T cells, PS exposure serves as a recognition marker for the ingestion of dying cells by phagocytes (Martin et al., 1995b), a process which needs to precede cell lysis in order to avoid an inflammatory response.

In order to establish the annexin-FITC assay for use in our T cell system, dose response and time course experiments were performed. One striking advantage of

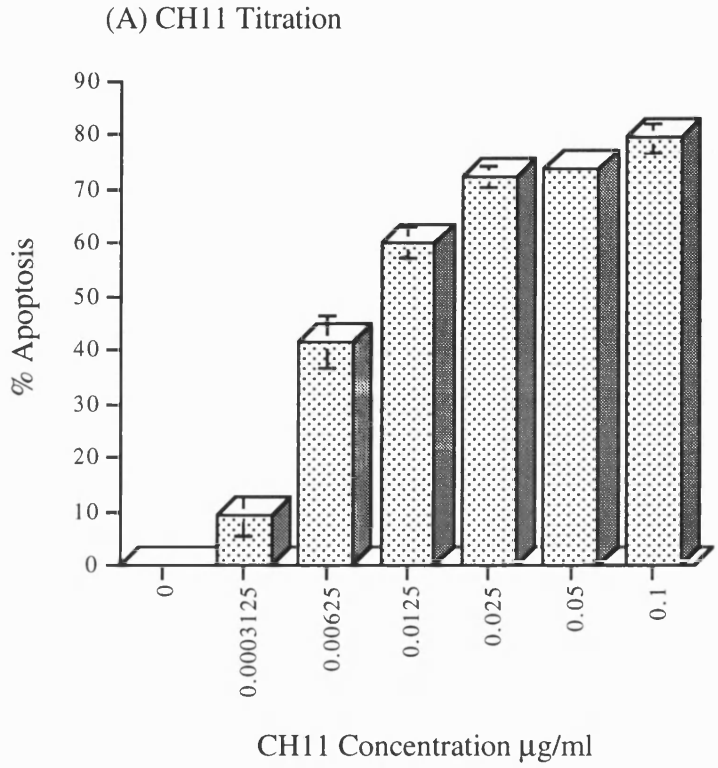
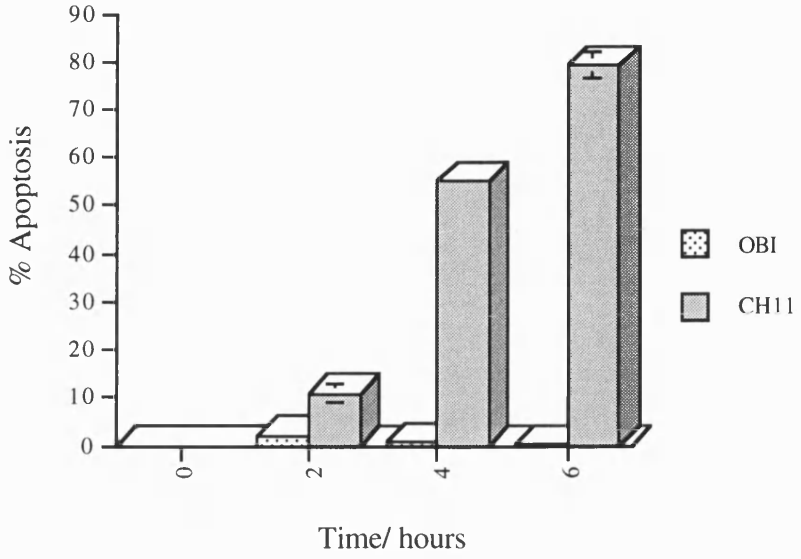
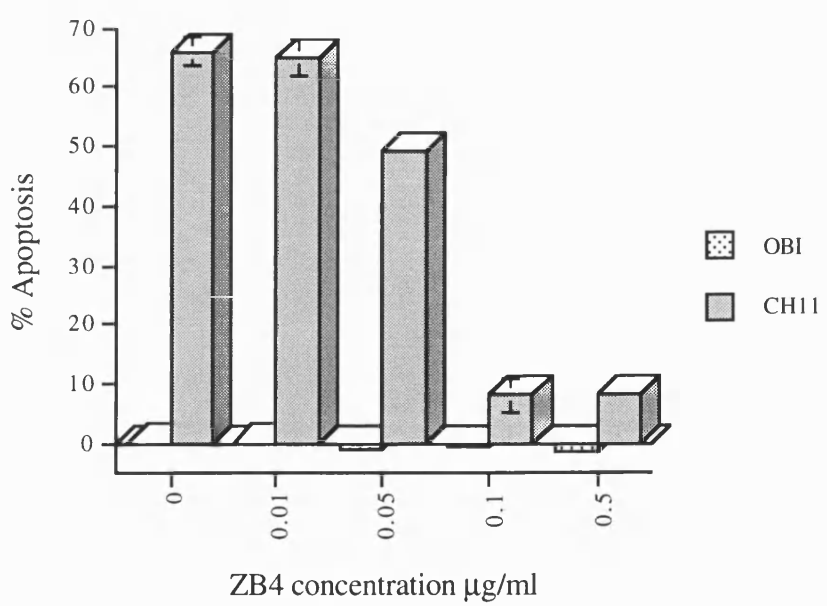


Figure 4.2: Establishment of the JAM assay for apoptosis measurement. ^{3}H-thymidine-labelled Jurkat cells were incubated as indicated, harvested and assayed by liquid scintillation counting. Apoptosis is calculated as % decrease CPM relative to control-treated Jurkats. (A) J16 cells were incubated for 15h with the indicated concentrations of the apoptotic anti-CD95 antibody CH11. (B) Cells were treated with CH11 (0.1µg/ml) or a control IgM antibody (OBI, supernatant) for the indicated time period. (C) OBI or CH11 (0.05µg/ml) treated cells were incubated for 15h with the blocking anti-CD95 antibody ZB4 at the indicated concentrations. Data indicate the mean (+/-SEM) of triplicate wells, and each experiment was repeated 3 times with similar results.

(B) CH11 Time Course



(C) ZB4 Titration



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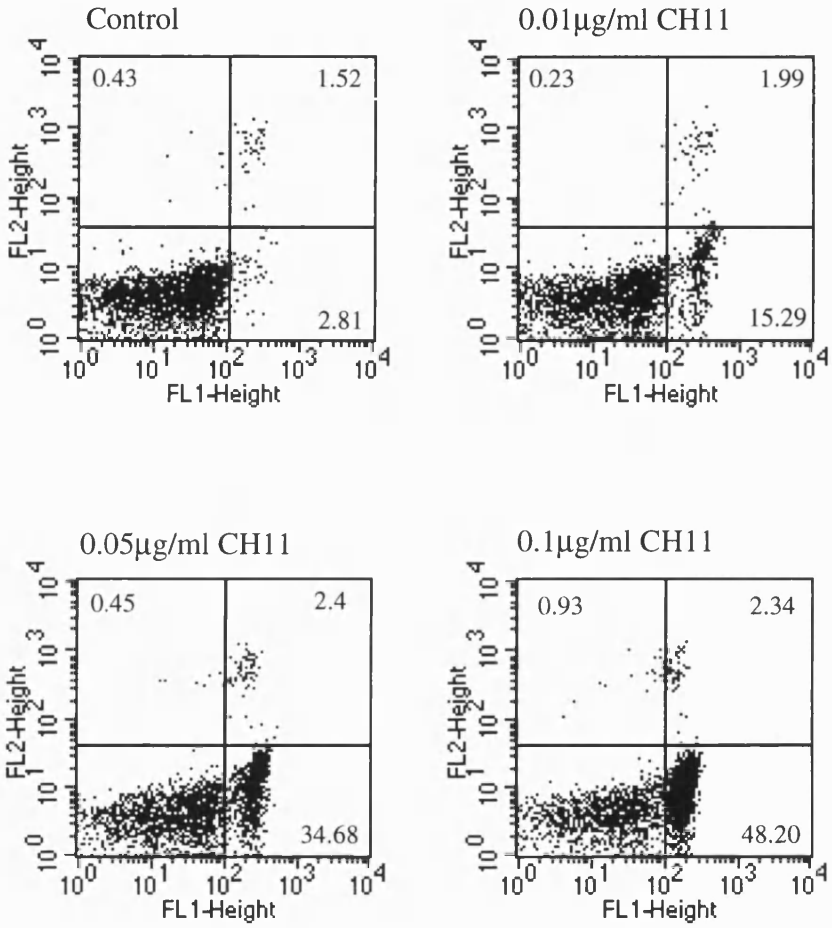


Figure 4.3: Dose-dependent CH11-induced apoptosis measured by the annexin-FITC binding assay. J16 cells were exposed to the indicated concentrations of the apoptotic anti-CD95 antibody CH11 for 4h then assayed for annexin-FITC binding and analysed by FACS. The control sample was incubated in the absence of CH11. Data are representative of 3 similar experiments.

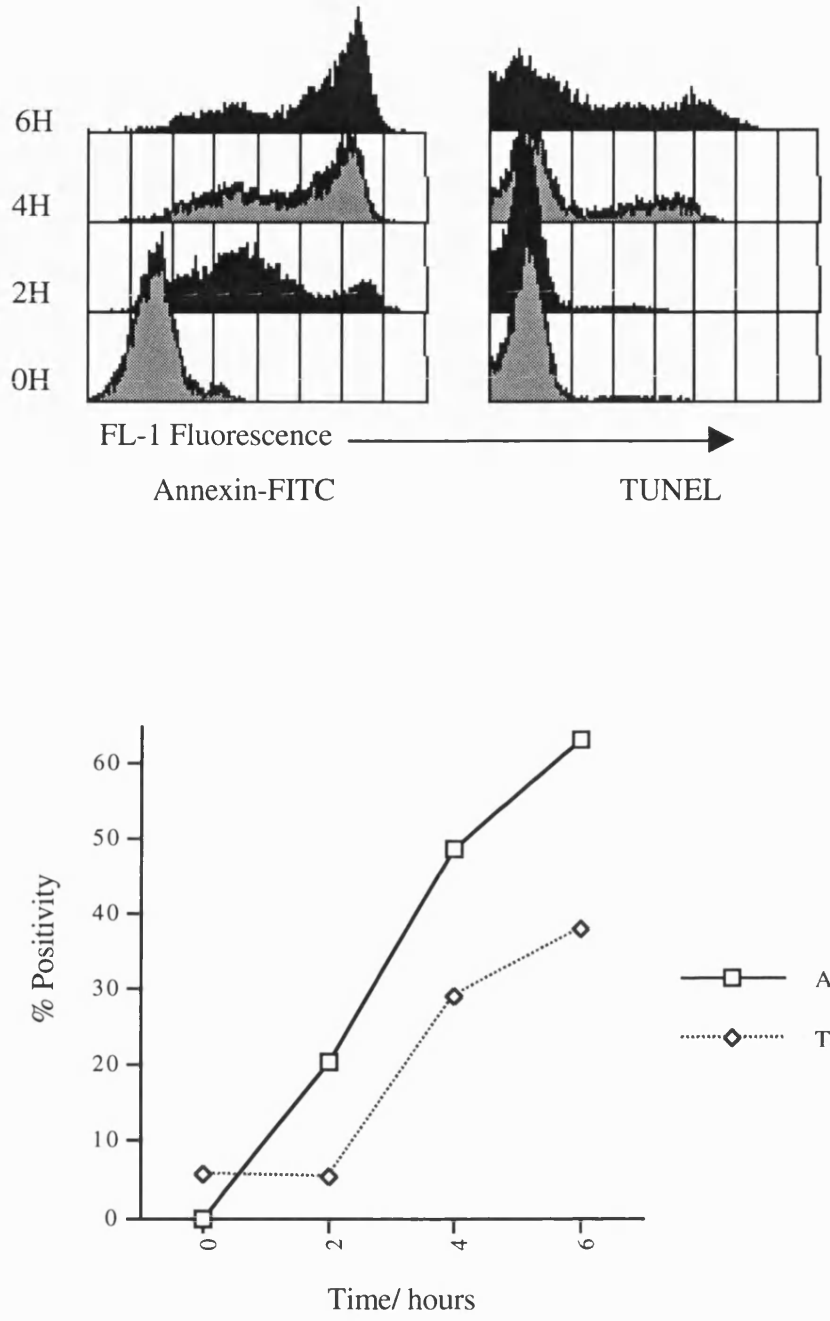


Figure 4.4: Apoptosis measurement using TUNEL analysis and annexin-FITC binding. J16 cells were incubated with the anti-CD95 antibody CH11 (0.05µg/ml) for the indicated time periods then assayed for apoptosis using annexin-FITC binding or TUNEL analysis, with increased fluorescence being visualised by FACS. The % of apoptotic cells at each time point is presented graphically in the lower panel. Data are representative of 2 similar experiments.

using FACS based assays was the ability to acquire data separately for individual cells which could then be collated into dot-plot form. Figure 4.3 illustrates apoptosis induction in J16 cells in response to a 4 hour incubation with the indicated concentration of the anti-CD95 antibody CH11 as measured by the annexin-FITC assay. The x axis represents annexin-FITC binding whilst on the y axis plasma membrane integrity was assessed by PI uptake. During the course of an apoptotic response, therefore, cells move from the lower left quadrant to the lower right (annexin bright) region and finally undergo secondary necrosis and occupy the upper right portion of the dot-plot. Since this was a short term experiment (4 hours), the cells largely retained membrane integrity and thus accumulated in the lower right ("early apoptosis") region of the dot plot. These data demonstrated the annexin assay to be a powerful and sensitive measurement of early apoptotic membrane changes.

The ability of the annexin-FITC assay to distinguish cells at an early time point during commitment to apoptosis was highlighted by a direct comparison with the TUNEL assay. Accordingly, the data shown in figure 4.4 reflect the temporal separation associated with the detection of membrane changes (by annexin-FITC binding) as compared to DNA fragmentation events (visualised by TUNEL). Therefore apoptotic cells could begin to be distinguished on the basis of their PS exposure only 2 hours after treatment with CH11, whilst DNA fragmentation was not apparent by TUNEL until at least 4 hours post stimulation. Thus a number of assays were established to detect different events which occurred during apoptosis.

4.2.2 Generation of soluble CD95L and a CD95-insensitive Cell Line

Since the use of monoclonal antibodies may not precisely mimic the effects of natural ligand, steps were taken to generate a source of soluble CD95L as an

additional tool with which to probe T cell responses to CD95 ligation. Details of the soluble CD95L production are presented in the methods section. Briefly, a CD95L cDNA clone was generated comprising the extracellular portion of CD95L fused to a marker epitope (FLAG, Kodak) with the addition of the CD80 signal sequence to target the resultant protein for secretion. The resulting construct was cloned into the eukaryotic expression vector pCDNA3 which contained a neomycin resistance gene and this vector was transfected into COS-7 cells by electroporation. The production of the CD95L-flag construct was carried out by Dr Yusuf Patel and the transfection of COS-7 cells with this construct was performed by Dr Julie McLeod working in our laboratory. A stable line was generated by culture in the presence of neomycin to select for cells expressing the vector. Cells were then cloned into 96 well plates and supernatants were subsequently screened for cytotoxic activity in the JAM assay using J16 cells. As indicated in figure 4.5 (A), a small number of colonies exhibited cytotoxic potential, and these clones were therefore selected and expanded in culture. Following expansion, the supernatants were harvested, filtered and tested in the JAM assay for the ability to induce CD95-dependent apoptosis. As a control, supernatant from untransfected COS cells was also tested for cytotoxicity in the JAM assay. Figure 4.5 (B) illustrates that the apoptotic effect of the resultant CD95L-containing supernatant was dose-dependent and occurred only in supernatants from transfected cells. In addition, CD95L-induced apoptosis was inhibited in the presence of CD95-specific blocking reagents (CD95-Fc), but not a control blocking reagent (CD23-Fc), ruling out the possibility of non-specific toxicity (figure 4.6, panel A).

As a further tool for these studies, a 'CD95-resistant' Jurkat cell line was also generated. To achieve this, J16 cells were subjected to sequential treatment with the apoptotic anti-CD95 antibody CH11. After each round of selection, surviving cells were expanded in culture prior to further treatment with augmented titres of CH11. The resultant cell line (JLW) retained equivalent surface CD95 expression

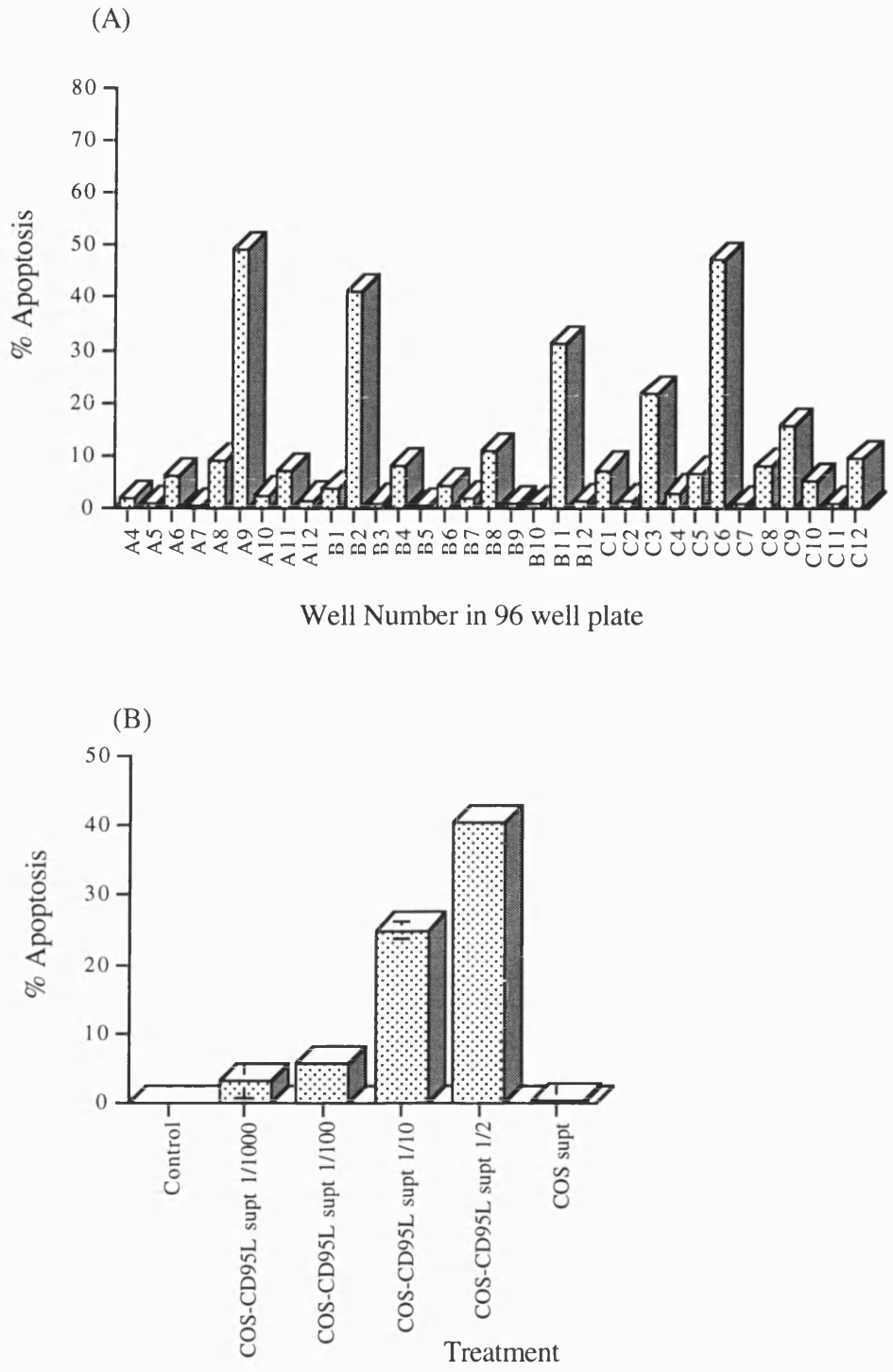


Figure 4.5: Production of soluble CD95L. (A) COS-7 cells transfected with CD95L were cloned into 96 well plates and screened for apoptotic activity against ^{3}H-thymidine-labelled Jurkats in a 15h JAM assay. (B) Supernatants from selected colonies were titrated in a 6h JAM assay. Supernatant from untransfected COS-7 cells was included as a control and apoptosis is presented as % death relative to control-treated Jurkats. Data in panel (B) indicate the mean (+/-SEM) of triplicate wells and this experiment was repeated twice with similar results.

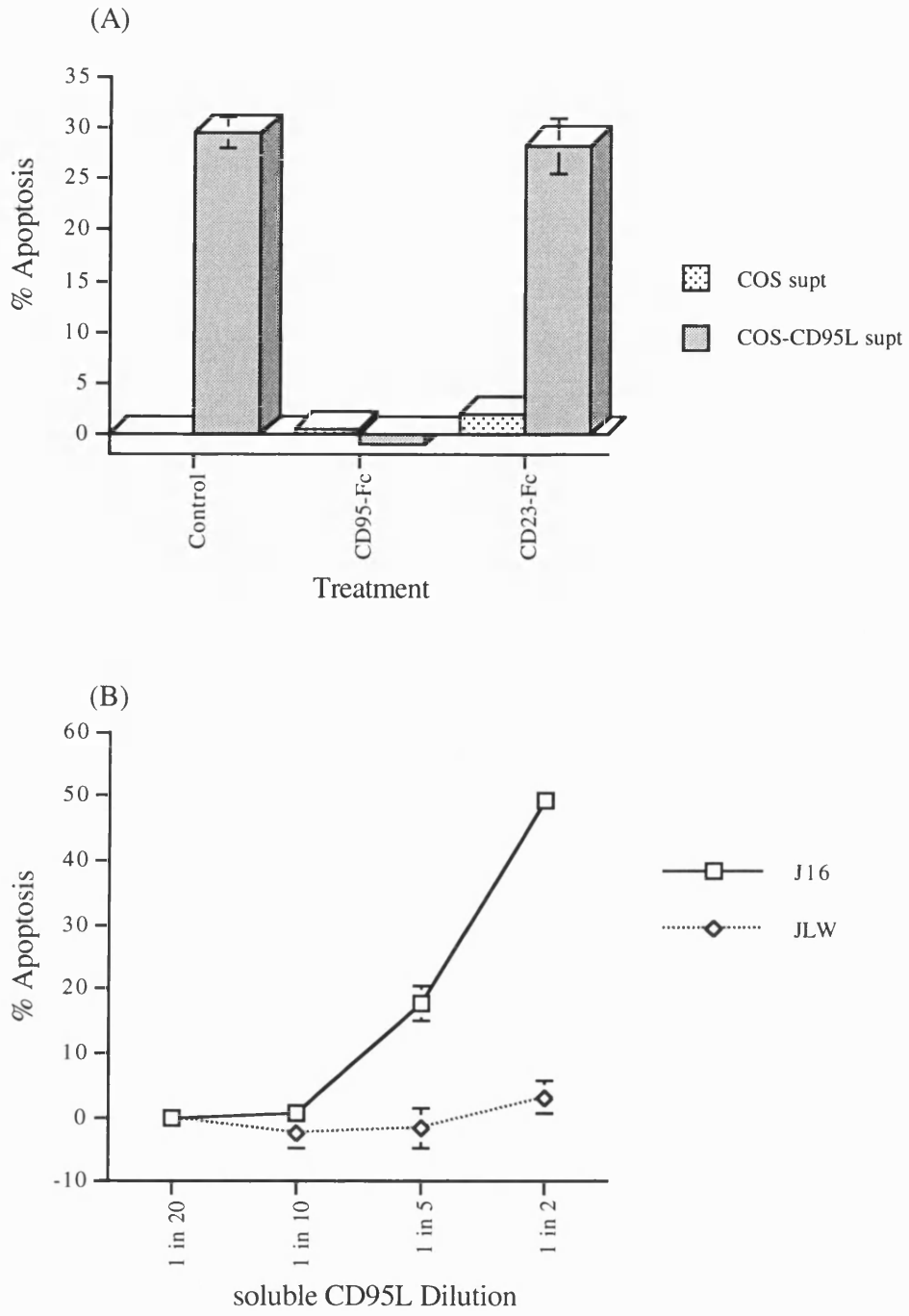


Figure 4.6: Analysis of the specificity of soluble CD95L using CD95-Fc and a "CD95-insensitive" cell line (JLW). (A) ^{3}H-thymidine-labelled J16 cells were incubated with supernatants from transfected (CD95L) or untransfected COS-7 cells in the presence of CD95-Fc or a control Fc construct (CD23-Fc). (B) JLW cells were derived from J16 by selection with apoptotic anti-CD95 antibody (CH11). J16 and JLW cells were ^{3}H-thymidine labelled and exposed to the indicated dilution of soluble CD95L. Apoptosis was measured by JAM assay and is presented as % death relative to control-treated Jurkats (mean +/-SEM of triplicate wells).

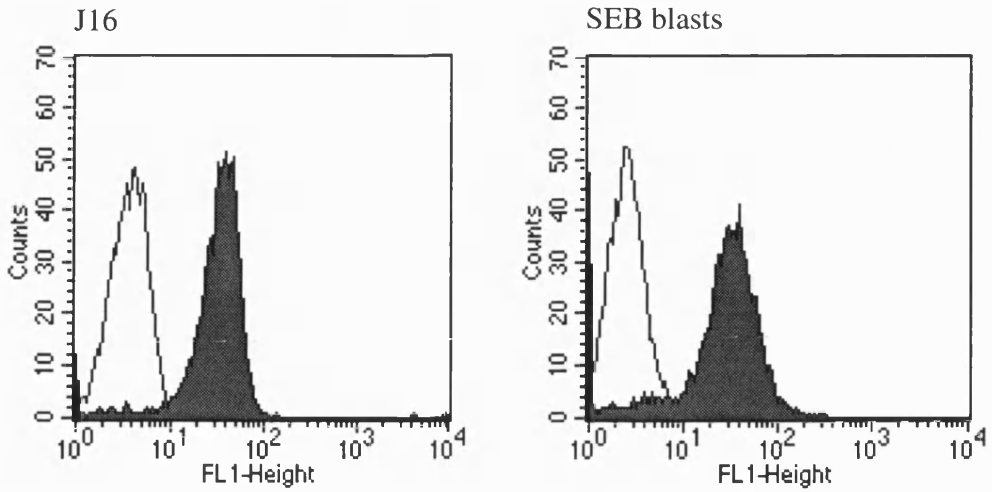
to the parental line, but nevertheless exhibited a marked reduction in the apoptotic response to CD95 ligation as illustrated by treatment with soluble CD95L (figure 4.6, panel B). This cell line proved useful in bioassays designed to dissect the cytotoxic mechanisms utilised by T blasts as presented in chapter 5.

4.2.3 Study of CD95 in Normal Activated T Cells

Having established that T cells could be effectively activated using the *in vitro* protocols described earlier, and having established assays for apoptosis measurement using Jurkat T cells, it was then possible to examine CD95 receptor engagement in normal peripheral blood T cells. Since Jurkat T cells readily exhibited CD95-induced apoptosis, these cells served as a useful control for the studies on normal T lymphocytes. Initial comparisons between J16 cells and normal T cell blasts on day 6 after activation (SEB blasts) indicated equivalent expression of the CD95 receptor as detected by surface staining and FACS analysis (figure 4.7, panel A). However, measurement of apoptosis induction using the JAM assay revealed a striking difference in the response to CD95 ligation between these two cell types (figure 4.7, panel B). Whilst the majority of J16 cells were apoptotic following a 15 hour incubation with the stimulating anti-CD95 antibody CH11 (0.05μg/ml), SEB blasts in contrast exhibited only a small decrease in viability as measured by DNA fragmentation in the JAM assay.

Since the viability of SEB blasts did not appear to be greatly compromised by incubation with CH11, the effect of different concentrations of this antibody was investigated. Previously presented data from J16 cells (figure 4.2, A) indicated maximal killing was achieved at approximately 0.025μg/ml CH11 under these conditions, and this dose induced apoptosis with equivalent potency and kinetics in a number of other human T cell lines examined (including HUT78). However, since SEB blasts appeared less sensitive to the effects of CD95 ligation, titration

(A) CD95 Expression



(B) CD95 Sensitivity

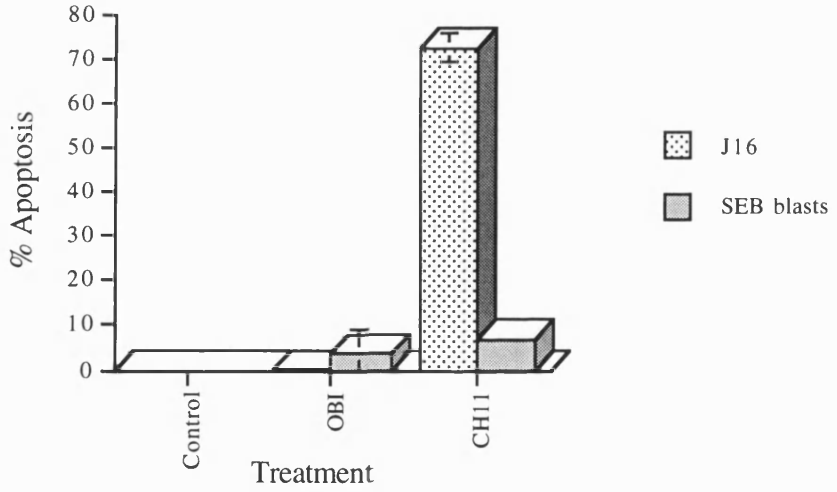


Figure 4.7: Resistance to CD95-induced apoptosis in SEB blasts compared to Jurkat cells despite equivalent CD95 expression. (A) Cells (J16 or day 6 SEB blasts) were stained for surface expression of CD95 (filled histograms). Open histograms indicate staining with secondary antibody only. (B) ^{3}H-thymidine-labelled cells from the same cultures as (A) were treated for 15h with OBI (supernatant) or the apoptotic anti-CD95 antibody CH11 (0.05µg/ml) in the JAM assay. Apoptosis is indicated as % death relative to control-treated cells (mean +/- SEM of triplicate wells). Data are representative of 5 experiments.

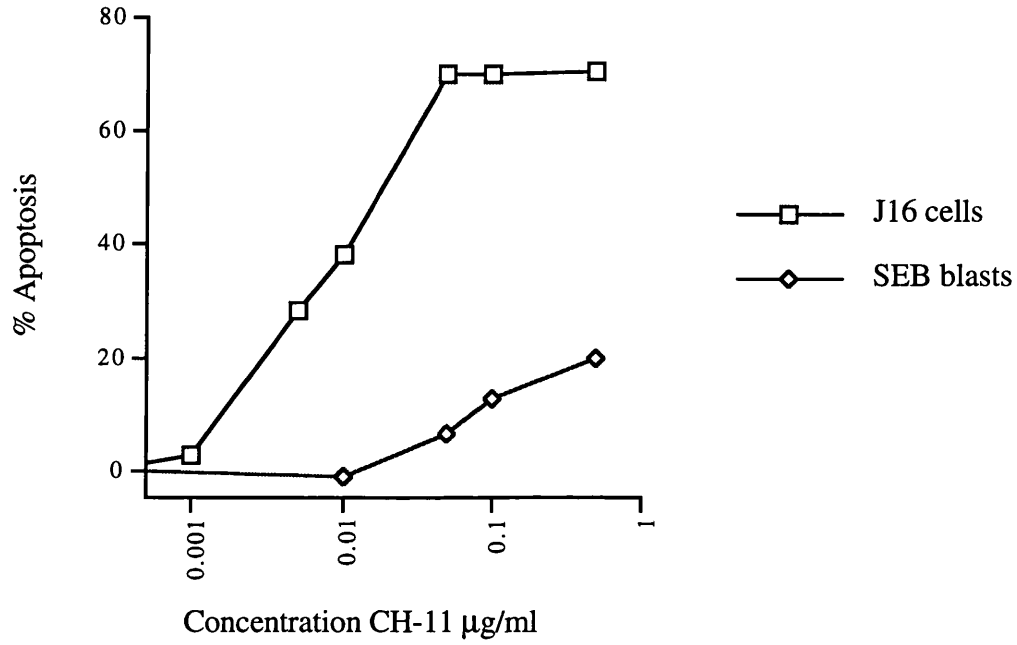


Figure 4.8: Dose response to CH11 in J16 cells and SEB blasts. ^{3}H-thymidine-labelled cells (J16 or day 6 SEB blasts) were exposed to the indicated concentration of the apoptotic anti-CD95 antibody CH11 for 15h in the JAM assay. Apoptosis is indicated as % death relative to control treated cells (mean +/- SEM of triplicate wells). These data are representative of 3 similar experiments.

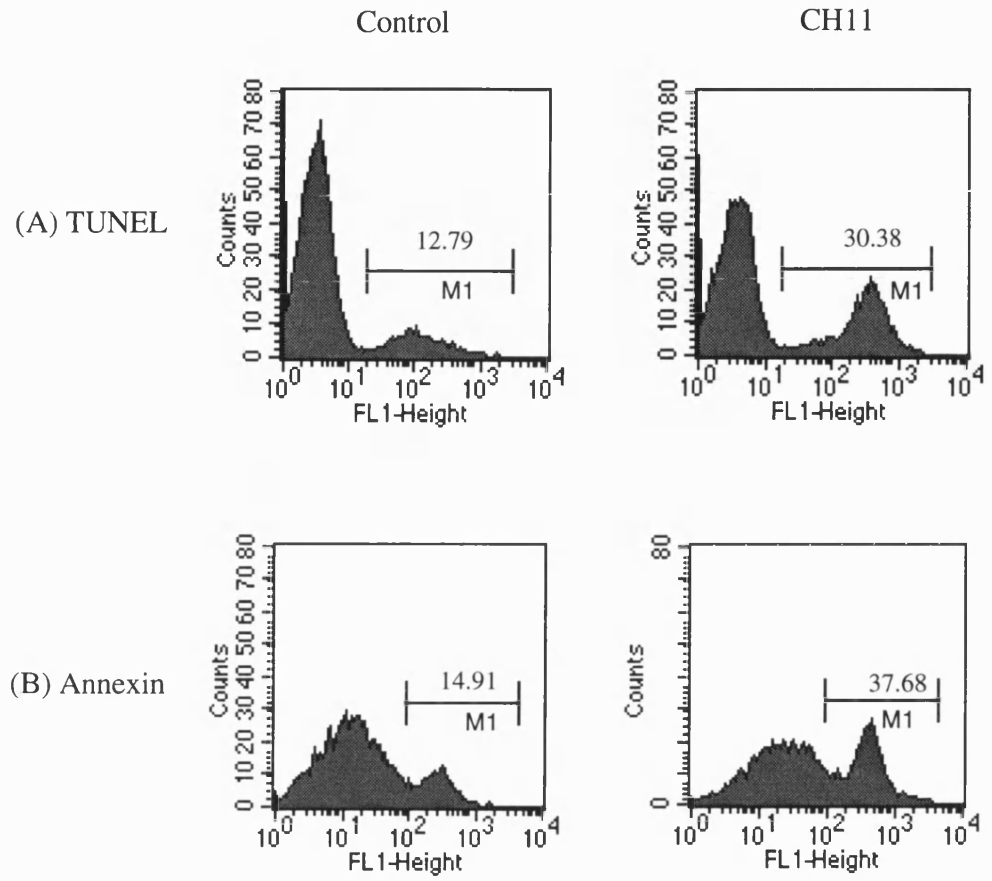


Figure 4.9: Resistance to CD95-mediated apoptosis in SEB blasts as measured by TUNEL and annexin-FITC analysis. SEB blasts (day 6) were treated for 15h with the apoptotic anti-CD95 antibody CH11 (0.5µg/ml) then assayed for apoptosis using the TUNEL assay (A) or annexin-FITC binding (B). Apoptosis is measured as an increase in fluorescence in the gated region of the histogram. The CH11-dependent increase in fluorescence is 17.59% as measured by TUNEL and 22.77% as measured by annexin-FITC binding in this experiment. Data are representative of 5 independent experiments.

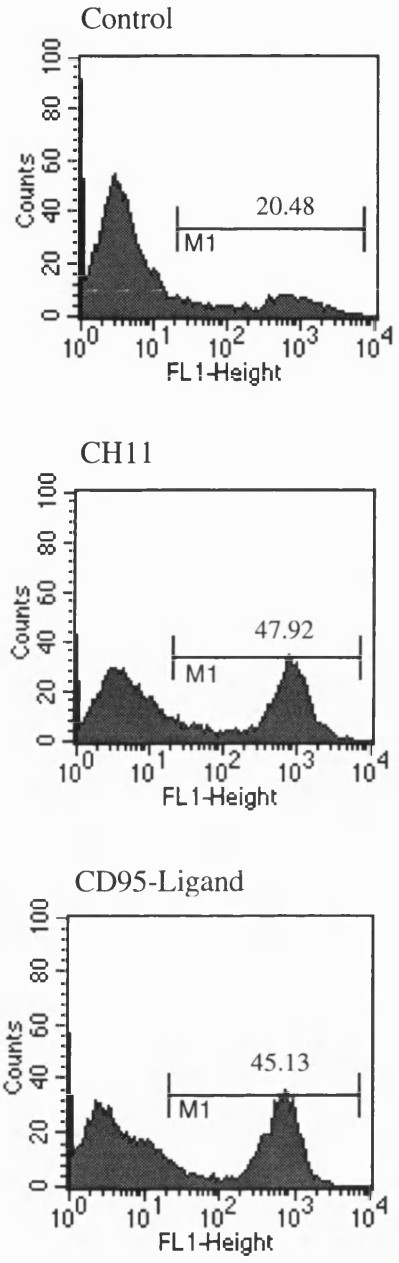


Figure 4.10: Comparison of antibody- and ligand-mediated CD95-induced apoptosis in T blasts. SEB blasts (day 6) were incubated for 15h with the anti-CD95 antibody CH11 (0.5µg/ml) or soluble CD95L (COS-7 cell supernatant). Apoptosis was measured by annexin-FITC binding and visualised as an increase in fluorescence by FACS (27.44% increase for CH11 treatment, 24.65% increase for soluble CD95L). Data indicate the maximum apoptotic response from 4 experiments.

experiments were performed utilising greater concentrations in order to establish whether the resistance to apoptosis was a consequence of limiting antibody. The data presented in figure 4.8 illustrate a dose-response to CH11 for both J16 cells and SEB blasts in the JAM assay. At concentrations as low as 0.005μg/ml, there was a marked apoptotic response in the J16 cells which increased before plateauing at approximately 0.05μg/ml in this experiment. In contrast, whilst SEB blasts exhibited a small dose-dependent increase in apoptosis as the CH11 concentration increased, the response was substantially lower than that seen in Jurkat cells. The fact that Jurkat cells exhibited maximal sensitivity to apoptosis at these doses of CH11, and that SEB blasts expressed equivalent surface CD95 receptor as detected by FACS analysis, indicated that there was sufficient antibody availability in these experiments to trigger apoptosis in susceptible cells.

To confirm that CD95 resistance in SEB blasts was not an artefact associated with use of the JAM assay for apoptosis measurement, alternative apoptosis assays were also used. Again, the restricted response to CD95 ligation in SEB blasts was confirmed regardless of the methodology employed: apoptosis following CH11 treatment was confined to a minority of the SEB blast population as measured by both annexin-FITC binding and TUNEL analysis (figure 4.9), even when using a relatively high concentration of CH11 (0.5μg/ml). Furthermore, apoptosis induction using soluble CD95L as an alternative to antibody-mediated ligation gave a similarly limited apoptotic response (figure 4.10), demonstrating that the resistance to apoptosis in SEB blasts was not simply an artefact associated with the anti-CD95 antibody CH11, but was a reproducible finding.

Since the surface marker expression and functional responses of T cells change during the activation process (chapter 3), the relationship between extent of cellular activation and resistance to CD95-mediated apoptosis was investigated. Accordingly, parallel analysis of CD95 receptor surface expression and sensitivity to CD95 crosslinking with CH11 was undertaken over the 7 day period following

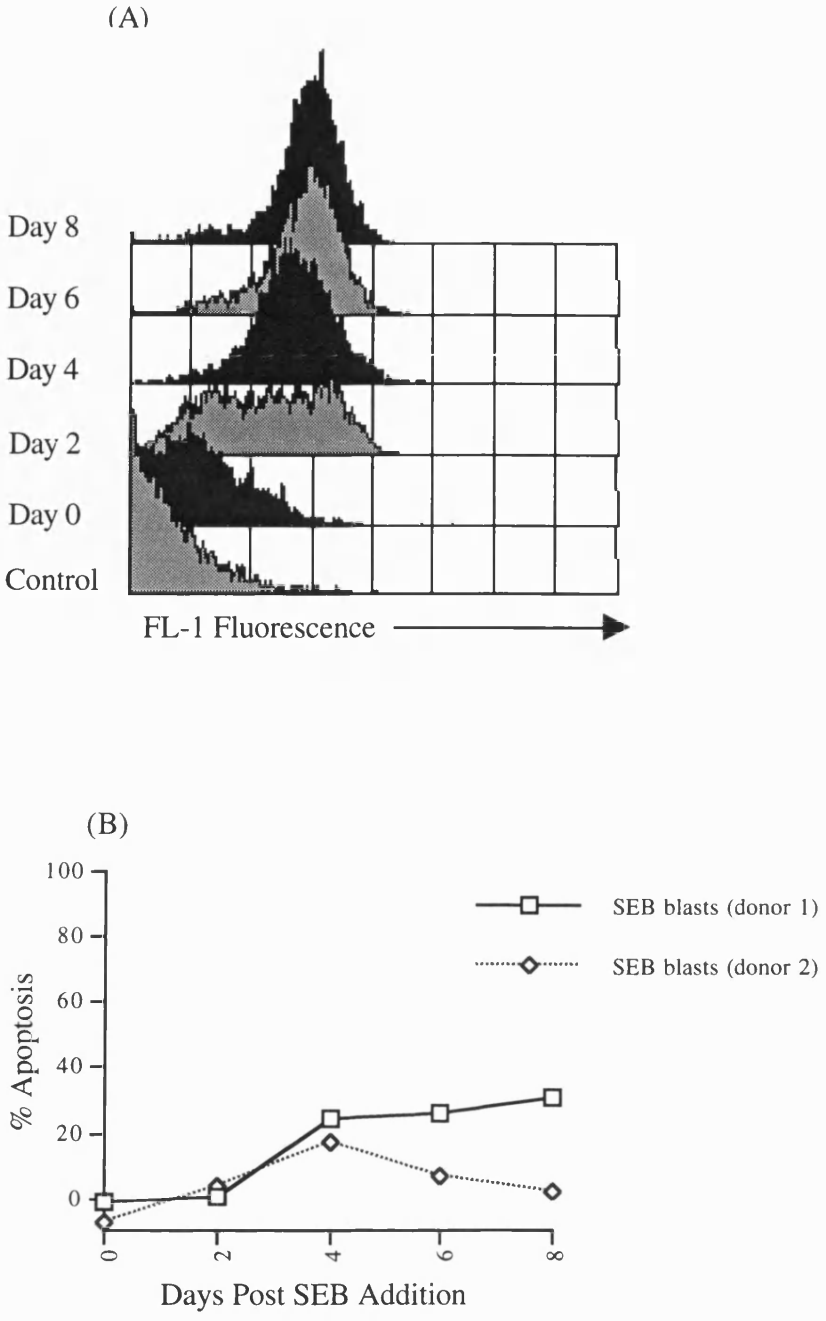


Figure 4.11: CD95 expression and CD95 sensitivity of human peripheral blood T cells during superantigen stimulation. Human PBMC were treated with SEB (1µg/ml) on day 0 and assayed for CD95 receptor expression (A) and CD95-induced apoptosis (B) at the indicated time points. Control staining reflects use of the secondary antibody only. (B) Apoptosis was induced by a 15h incubation with the anti-CD95 antibody CH11 (0.5µg/ml) and was measured by TUNEL assay. Data are presented as % CD95-induced death relative to control treated cells and this experiment was repeated using 6 different donors with similar results.

superantigen stimulation. As shown in figure 4.11, the CD95 receptor was upregulated during the first 4 days of activation (panel A) and the sensitivity to apoptosis via this route increased accordingly (from 0% to approximately 20%) (panel B). After this time point, however, despite high CD95 receptor expression, which was equivalent to levels found on J16 cells, the apoptotic response was nevertheless confined to a minority of cells (less than 35% at all time points) whereas J16 cells underwent apoptosis readily following CD95 ligation. These data therefore contrasted with previously published findings which demonstrated a substantial apoptotic response of human T cells at these time points (Owen-Schaub et al., 1992; Klas et al., 1993).

To rule out the possibility that resistance to CD95-mediated apoptosis was merely a feature of superantigen stimulation of cells, a number of alternative mitogenic treatments were utilised including PHA and ConA. As outlined in the introduction, these reagents are routinely used for the *in vitro* generation of activated T cells from resting PBMC and are competent to induce proliferation and the predicted surface marker changes. A comparison of day 6 activated T cells generated by use of the above stimulations revealed that substantial CD95 resistance, as measured by TUNEL assay, was common to all of these activated T cell cultures rather than being specifically associated with superantigen stimulation (figure 4.12).

Another possibility to be addressed was that T cell blasts may be protected from apoptosis by the serum used in their culture medium. Therefore to investigate this hypothesis, resting T cells from the same donor were cultured by superantigen stimulation either in routine medium (RPMI-1640 plus 10% FCS) or in serum free medium (AIM-V). As shown in figure 4.13, cells grown in AIM-V underwent the characteristic surface marker changes associated with T cell activation including upregulation of CD95 (A) and were able to proliferate to a comparable degree as cells grown in routine medium (B). These data indicated that effective T cell

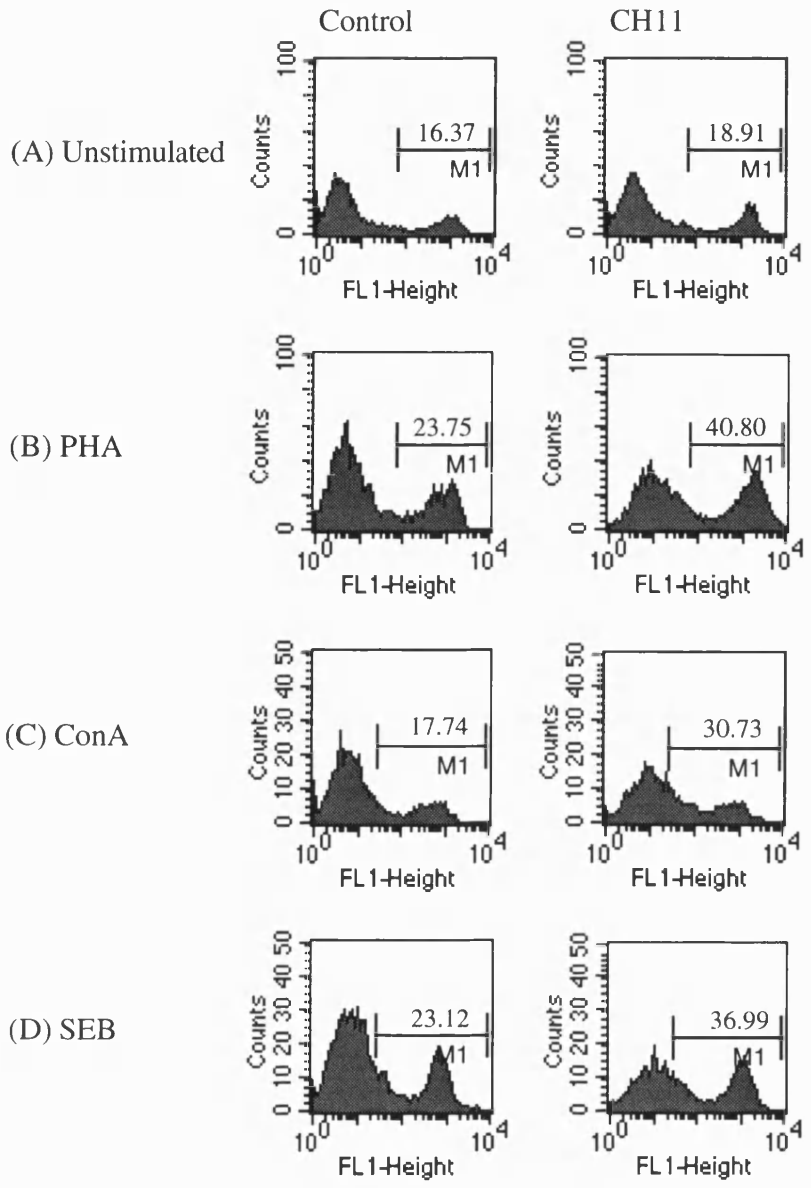


Figure 4.12: CD95-mediated apoptosis of human PBMC activated using diverse stimuli. Resting human PBMC were stimulated with PHA (2µg/ml), ConA (1µg/ml) or SEB (1µg/ml) for 6 days then treated with the anti-CD95 antibody CH11 (0.5µg/ml) for 15h. Apoptosis was measured by TUNEL analysis. CH11-specific increases in apoptosis of 2.54% (A), 17.05% (B), 12.99% (C) and 13.87% (D) were observed in this experiment. Data are representative of 3 independent experiments.

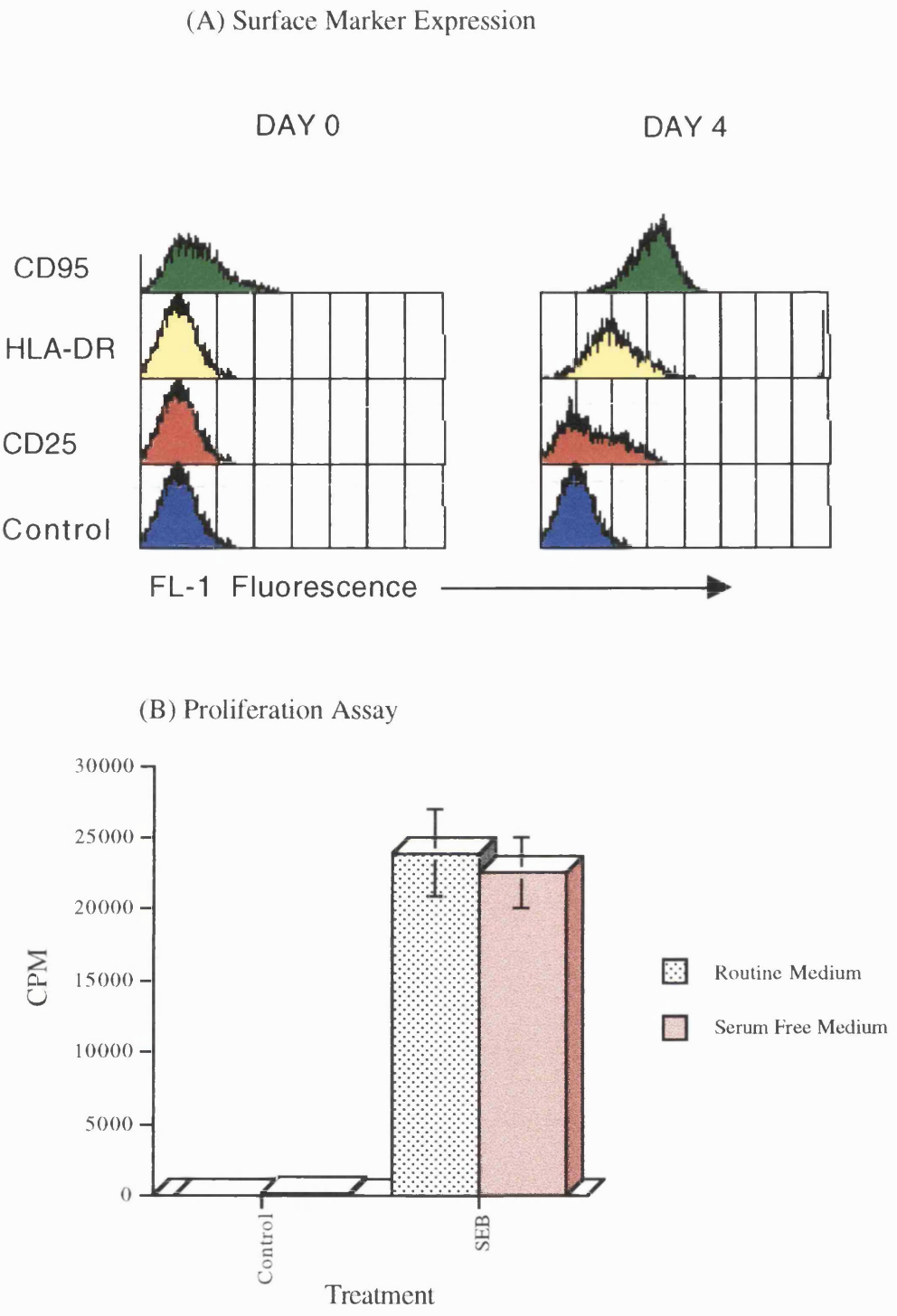


Figure 4.13: Effective activation of T cells stimulated in serum free medium. Human PBMC were stimulated with SEB (1µg/ml) in routine medium (RPMI +10% FCS) or serum free medium (AIM-V). (A) Surface expression of CD25, HLA-DR, and CD95 was assessed for cells cultured in serum free medium. Control staining indicates use of secondary antibody alone. (B) Proliferation of T cells (2 x 10^4 cells per well) stimulated in routine medium *versus* serum free medium was measured by ^{3}H-thymidine incorporation and are displayed as the mean of triplicate wells (+/- SEM). These data are representative of 2 similar experiments.

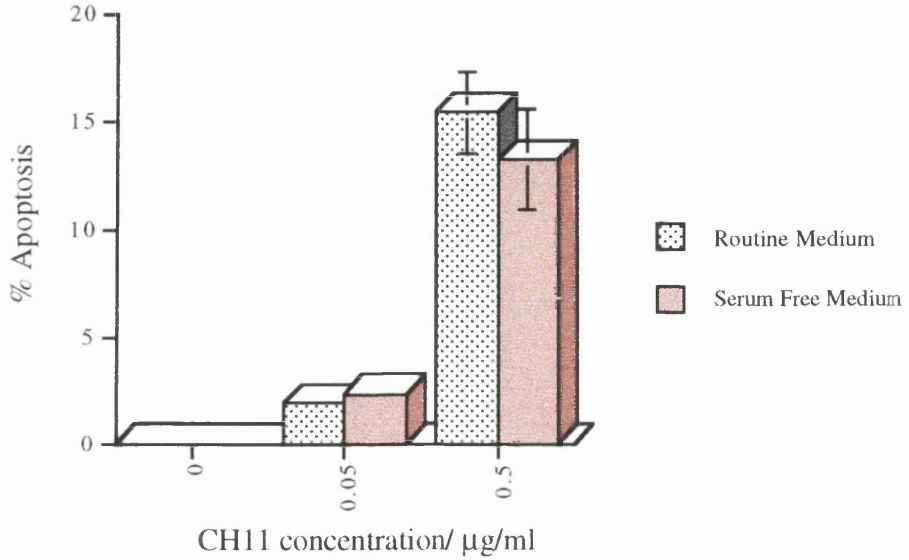


Figure 4.14: CD95-mediated apoptosis of T cells stimulated in serum free medium. Human PBMC were activated for 6 days with SEB (1µg/ml) in routine medium (RPMI + 10% FCS) or serum free medium (AIM-V) then labelled with ^{3}H-thymidine and treated for 15h with the indicated concentrations of the anti-CD95 antibody CH11. Apoptosis was measured by JAM assay and is calculated as % apoptosis relative to control treated cells. The mean (+/-SEM) of triplicate wells is shown. Data are representative of 2 experiments.

activation had occurred in cultures generated in serum free medium. The sensitivity to CD95-mediated apoptosis, as measured by JAM assay (figure 4.14), was also equivalent between these two cultures, indicating that the resistance to apoptosis exhibited by activated T cells was not associated with protective factors present in the FCS.

To examine whether the resistance to apoptosis in SEB blasts was mediated by a soluble factor, supernatants from activated T cell cultures were tested for their ability to inhibit CH11-mediated apoptosis of J16 cells in the JAM assay. One possibility was that a soluble version of the CD95 receptor might be produced by these cells and might act as a competitive inhibitor for CD95L/CH11 binding, and indeed it has been demonstrated that soluble CD95 proteins can be generated by alternative splicing (Cheng et al., 1994; Cascino et al., 1995). However, as indicated in figure 4.15, supernatants from activated T cells were unable to inhibit CD95-mediated apoptosis of J16 cells, suggesting that resistance was not transferable by a soluble factor.

Since the apoptotic response appeared to be restricted to a subpopulation of cells, one possibility was that sensitivity to CD95 may be cell cycle dependent. If, for example, S-phase entry was a requirement for the acquisition of sensitivity to CD95, then this might explain the limitation on apoptosis induction in these cultures. Consistent with this hypothesis, it has been previously suggested that cell cycle status may influence apoptosis sensitivity in normal human T cells (Lenardo, 1991; Boehme and Lenardo, 1993). To assess this, simultaneous cell cycle analysis and apoptosis measurement by TUNEL was performed both for J16 cells and SEB blasts. To visualise the cell cycle status of cells within a population, the DNA dye PI was used following ethanol permeabilisation of T cells. In the resulting histogram, the G0/G1 peak (comprising cells containing one copy of DNA) can be distinguished from S-phase (DNA synthesis phase) and G2 (two copies of DNA prior to cell division) phase cells, as indicated in figure 4.16

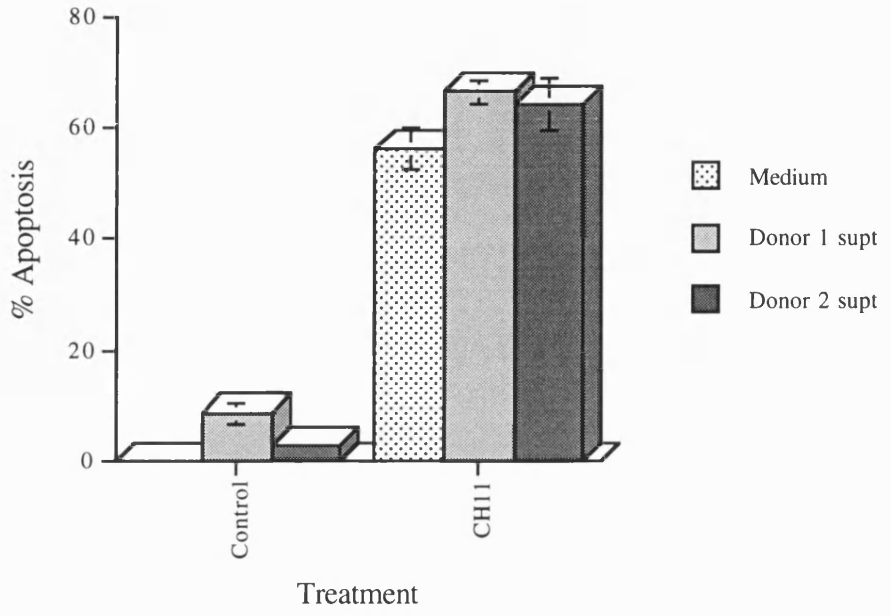


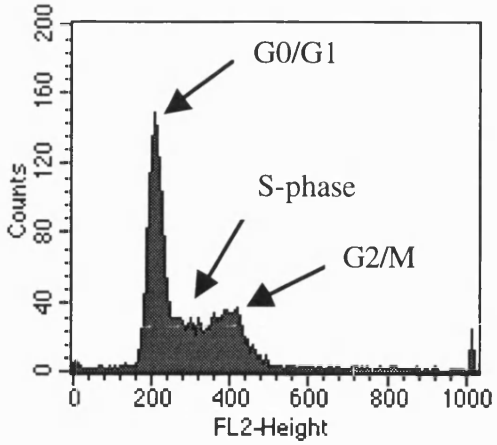
Figure 4.15: Effect of T blast supernatants on CH11-mediated apoptosis in J16 cells. Cell supernatants from day 6 SEB-activated (1µg/ml) human PBMC were tested for their ability to inhibit CD95-mediated apoptosis of J16 cells in the JAM assay. ^{3}H-thymidine-labelled J16 cells were incubated with the anti-CD95 antibody CH11 in the presence of T blast supernatants as indicated. Apoptosis was measured by JAM assay and is calculated as the % decrease in CPM relative to control-treated cells. Columns represent the mean (+/-SEM) of triplicate wells. Supernatants from SEB blast lines generated from 2 further donors were tested with similar results.

(A). By incorporation of TUNEL analysis into this protocol, the extent of apoptosis within each portion of the cell cycle histogram could be simultaneously assessed. Figure 4.16 (B) illustrates a viable J16 population denoted by contour plot, with the PI based cell cycle analysis plotted on the y axis in this case.

Application of this methodology to J16 cells (figure 4.17, A) indicated that, as expected, there was a strong apoptotic response to CD95 ligation, and interestingly this response was not restricted to one particular phase of the cell cycle since there was a reduction in both the upper left and lower left quadrants following CH11 treatment. In contrast, the SEB blast contour plots (figure 4.17, B) showed only a 9% increase in the TUNEL bright (right) quadrants following CD95 ligation indicating that, consistent with the previous data, the apoptotic response to CD95 engagement appeared to be subject to restrictions in activated peripheral blood T cells as compared with Jurkat T cell lines. Importantly, analysis of the cell cycle data revealed that rather than S-phase/G2 entry being a requirement for CD95 sensitivity, in fact there appeared to be a bias towards cells from the G0/G1 region of the histogram (lower left) exhibiting more apoptosis following CH11 treatment. Accordingly, the S-phase/G2 portion of the contour plot (upper left) was not reduced following exposure to anti-CD95, whilst the G0/G1 region (lower left) in contrast exhibited an 11% decrease following treatment. Since the majority of T blasts existed in this G0/G1 state (as visualised by cell cycle histograms), the restricted response to CD95 engagement did not merely reflect the proportion of cells which were transiting S-phase or were present in G2 phase at the time of the CD95 challenge.

Since an apoptotic response appeared to be favoured in those cells which were present in the G0/G1 peak, T blasts were allowed to quiesce in culture such that their viability was not substantially impaired, but they began to exit cell cycle. To achieve this, cultures were left without restimulation for 14-20 days in the absence of exogenous IL-2. In some cases this was severely detrimental to cell survival

(A)



(B)

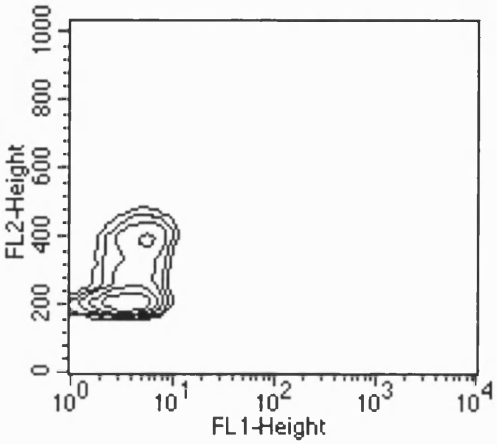


Figure 4.16: Cell cycle analysis by DNA staining with PI. (A) J16 cells were permeabilised with ethanol (15-18h, -20°C) and stained with PI (100µg/ml, 15min) to allow visualisation of DNA content. The peaks represent cells in different phases of cell cycle as indicated. (B) Simultaneous analysis of cell cycle and TUNEL positivity was performed as detailed in the methods section. Data was displayed as a contour plot allowing cell cycle to be measured on the y axis and TUNEL fluorescence on the x axis. This plot features a viable J16 population and thus lacks TUNEL positivity.

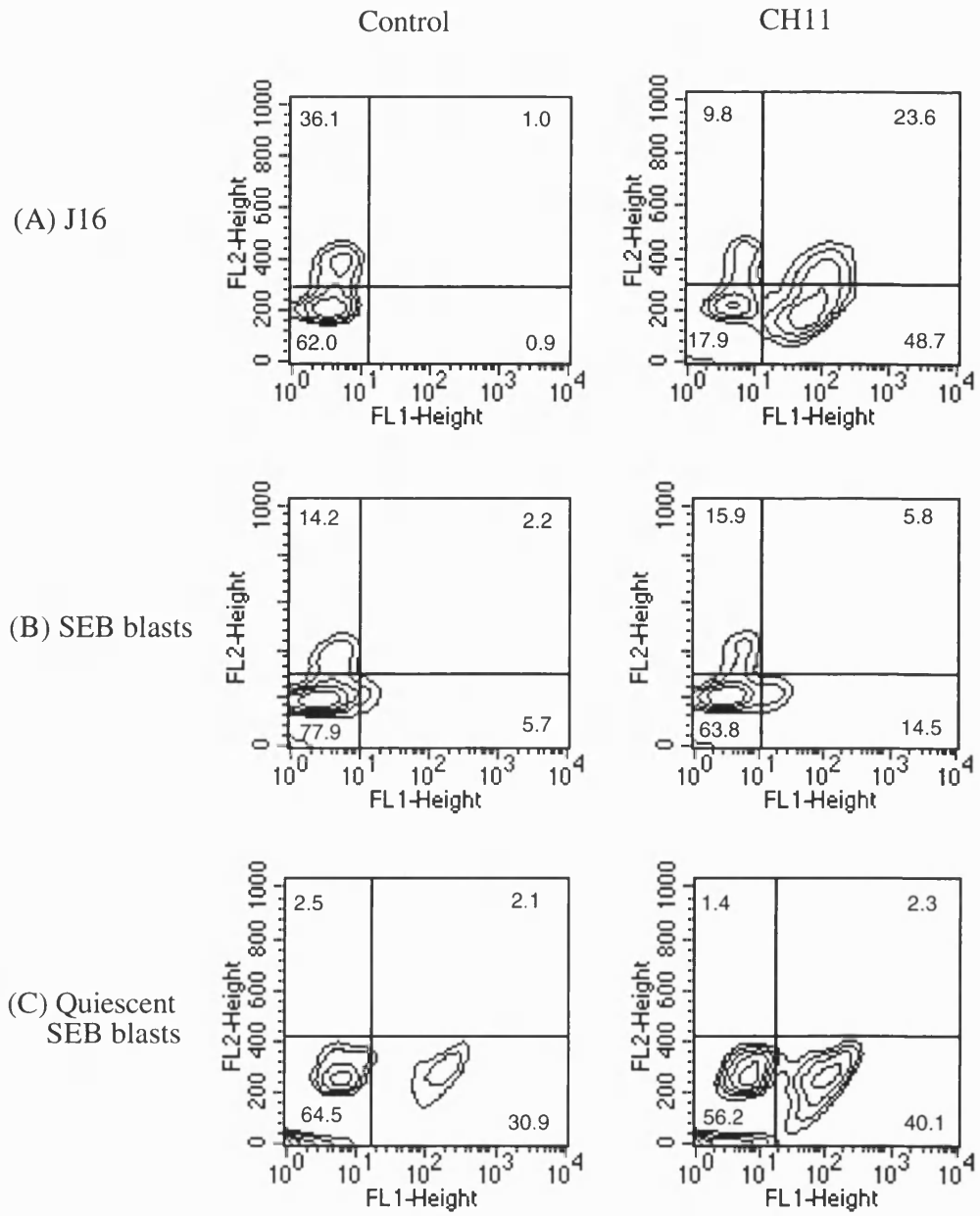


Figure 4.17. Dual staining for cell cycle status and apoptosis following CH11 treatment of T cells. J16 cells, day 6 SEB blasts or quiescent SEB blasts (day 17) were treated for 15h with the anti-CD95 antibody CH11 at 0.01µg/ml (A) or 0.5µg/ml (B) and (C). Simultaneous analysis of DNA content by PI staining (FL-2 fluorescence) and apoptosis by TUNEL assay (FL-1 fluorescence) was performed as described in the methods section. Data are representative of 3 experiments.

with overall viability dropping dramatically. However, on other occasions it proved possible to quiesce cultures in this manner with the result that DNA content histograms exhibited a near total absence of cells in S/G2-phase. This is illustrated by the PI cell cycle histogram shown in figure 4.17 (panel C) where the withdrawal from cell cycle can be clearly visualised by FL-2 fluorescence on the y axis. The data in this figure indicate that despite this lack of cycling, the proportion of cells sensitive to CD95-mediated apoptosis nevertheless remained low (9% kill as measured by TUNEL assay).

Since the responses of T cells may differ according to whether they exhibit a Th1 or Th2 profile of cytokine secretion (Mosmann and Coffman, 1989) it was possible that the subpopulation of SEB blasts which underwent apoptosis following CD95 ligation represented those displaying a certain Th phenotype. Therefore experiments were carried out in an attempt to elucidate whether there was a differential response to CD95 engagement depending on Th differentiation. In order to address this issue purified CD4+ T cells were activated with HLA-DR/CD80-presented superantigen (SEB) in the presence of cytokines which reputedly bias the nature of the Th response: either IL-12 to favour Th1 development, or IL-4 to bias towards a Th2 phenotype (Noble et al., 1993; Seder et al., 1993). Surface staining following purification confirmed that all detectable CD8+ cells had been successfully removed from these starting cultures (figure 4.18, A). Intracellular staining was then performed to assess which cytokines could be produced by these cultures. As indicated in figure 4.18 (panel B), IL-2 production was substantially increased in the "Th1"-like population, with IFNγ also being detected in these T blasts. In contrast, the "Th2"-like cells exhibited a reduced IL-2 output and undetectable IFNγ. Disappointingly, IL-4 was not detectable in either of these populations making it difficult to draw conclusions regarding the extent of differentiation achieved in these cultures. Whilst IL-4 is not a categorical marker for Th2 cells, some positivity would be expected from a population biased in this way. Thus whilst the differential IL-2 production is

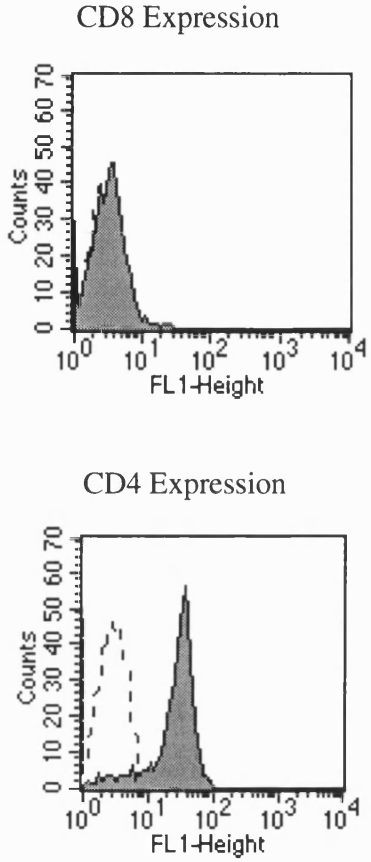


Figure 4.18 (A): Purification of CD4+ T cells. Resting human CD4+ T cells were purified from PBMC by immunomagnetic depletion. The resultant population was stained for surface expression of CD4 and CD8 (filled histograms). Dotted lines indicate control staining with secondary antibody only. Data are representative of the staining patterns observed each time CD4+ T cells were purified.

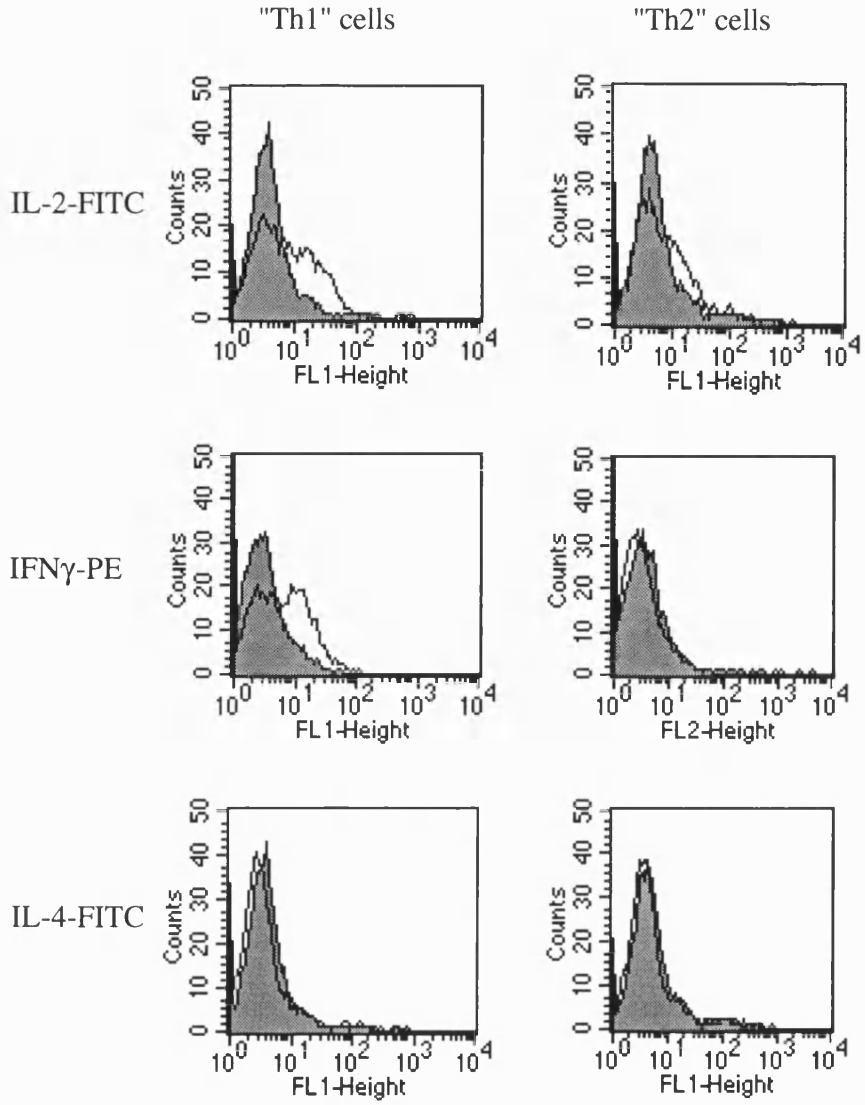


Figure 4.18 (B): Differentiation of CD4+ T cells by addition of exogenous cytokines. CD4+ T cells were activated with SEB-pulsed HLA-DR/CD80 transfectants in the presence of either exogenous IL-12 (0.5ng/ml) to favour Th1 differentiation or IL-4 (0.5ng/ml) to favour Th2 differentiation. Cells were restimulated in this manner every 10-14 days. Six days following the third stimulation, cells were stained for intracellular cytokine expression using FITC- or PE- conjugated antibodies (open histograms). Filled histograms indicate staining with isotype-matched control antibodies. Data are representative of 3 similar experiments.

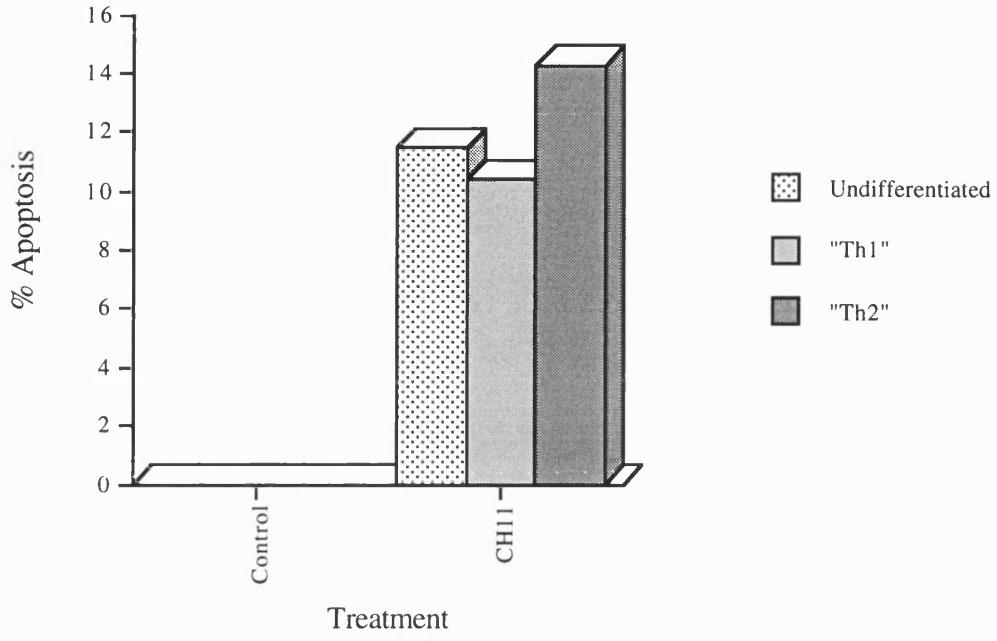


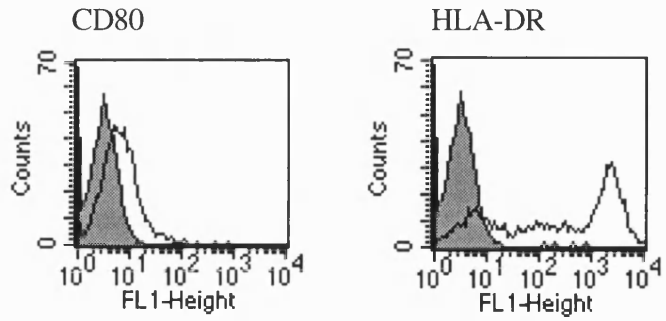
Figure 4.19: Sensitivity to CD95-induced apoptosis in "Th1"and "Th2" T cells. Resting CD4+ T cells were activated with SEB-pulsed HLA-DR/CD80 transfectants in the presence of IL-12 ("Th1" cells) or IL-4 ("Th2" cells). "Undifferentiated" indicates cells activated in the absence of exogenous cytokine. Cells were treated for 15h with the anti-CD95 antibody CH11 (0.5µg/ml) and apoptosis was measured by annexin-FITC binding and is calculated as % increase in fluoresence relative to control-treated cells. Data are representative of 3 experiments.

indicative of some measure of successful differentiation, the absence of a marker for "Th2"-like cells, renders firm conclusions difficult.

Since it appeared that the populations generated by the cytokine treatment described above exhibited some distinctions in terms of cytokine output, experiments were undertaken to determine whether there was a differential response to CD95 ligation in these cultures. Accordingly, samples from each population were treated with the anti-CD95 antibody CH11 and apoptosis was measured by annexin-FITC binding (figure 4.19). These data illustrated that there were no obvious differences in the sensitivity to CD95-mediated apoptosis between populations, with the apoptotic response being confined to a minority of cells in all cases. If a degree of T cell differentiation had indeed occurred in these populations, as indicated by the IL-2 and IFNγ staining data, then it had not affected the susceptibility to CD95-mediated apoptosis in these cells. Alternatively, polarised Th differentiation simply may not have occurred under these conditions and in the absence of a successfully tested marker for Th2 cell identification, these studies were not extended further.

As an alternative model for studying T cell responses, T cell clones which are long term cultures but which retain the requirement for antigen and costimulation in order for proliferation to be maintained were also used. Since normal activated T cells appeared to be restricted in their response to CD95 ligation, whilst Jurkat T cells were constitutively sensitive to apoptosis via this route, the relative sensitivity of T cell clones to this form of cell death was also assessed. The alloreactive T cell line 7418 was utilised for these studies and restimulated every 10-17 days using the B cell line ROF (expressing CD80 and HLA-DR) (figure 4.20, panel A) and irradiated PBMC. The latter are routinely used in the culture of T cell clones for the provision of undefined stimulatory ligands/cytokines, with the irradiation process being necessary to prevent their own proliferation. The successful stimulation of this T cell clone under the conditions described is

(A) Surface Staining of ROF Cells



(B) Stimulation of T cell clones with ROF cells

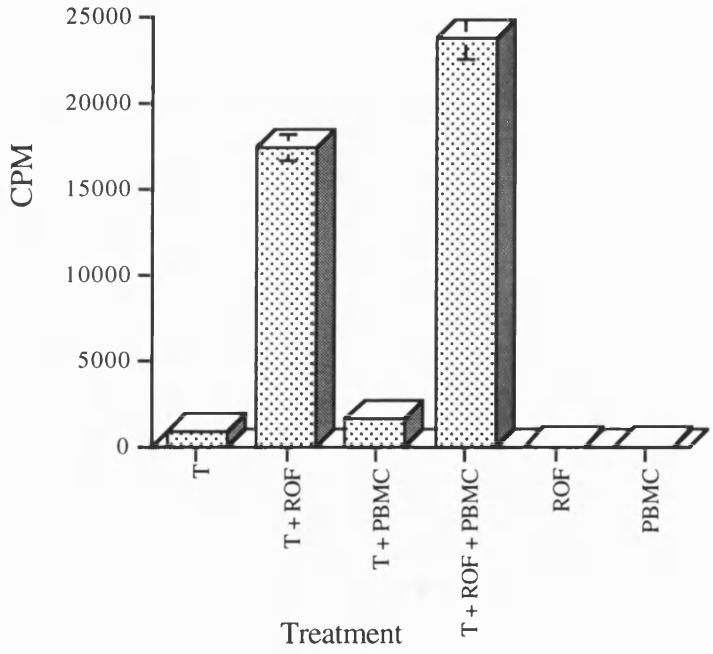
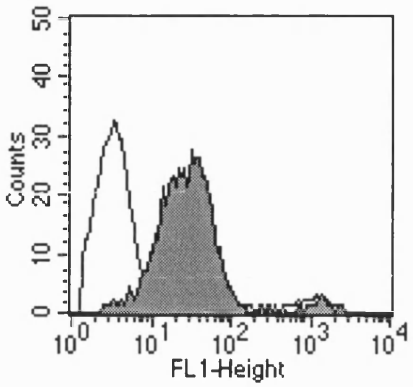


Figure 4.20: ROF-dependent proliferation of alloreactive T cells. (A) ROF B cells were stained for surface expression of CD80 or HLA-DR (open histograms). Filled histograms indicate control staining with secondary antibody alone. (B) 7418 alloreactive T cells were treated with fixed ROF cells (ratio 5:1 T cell:ROF cell), and irradiated PBMC (ratio 1:1 T cell:PBMC) as indicated. Proliferation was assessed by ^{3}H-thymidine incorporation and the mean (+/-SEM) of triplicate wells is shown. Data are representative of 3 experiments.

(A) CD95 Expression



(B) CD95-induced Apoptosis

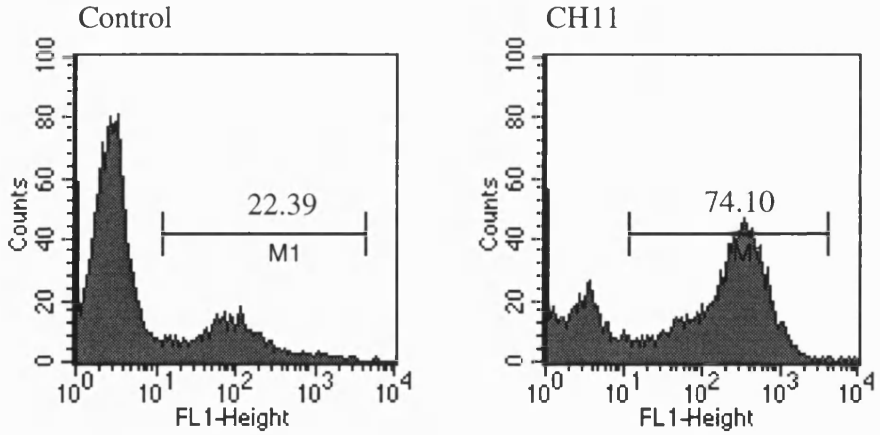


Figure 4.21: CD95 expression and CD95 sensitivity in alloreactive T cells. (A) 7418 alloreactive T cells were stained for CD95 expression. (B) T cells were incubated for 15h with the anti-CD95 antibody CH11 (0.5µg/ml) and apoptosis was measured by annexin-FITC binding. Data are representative of 4 separate experiments.

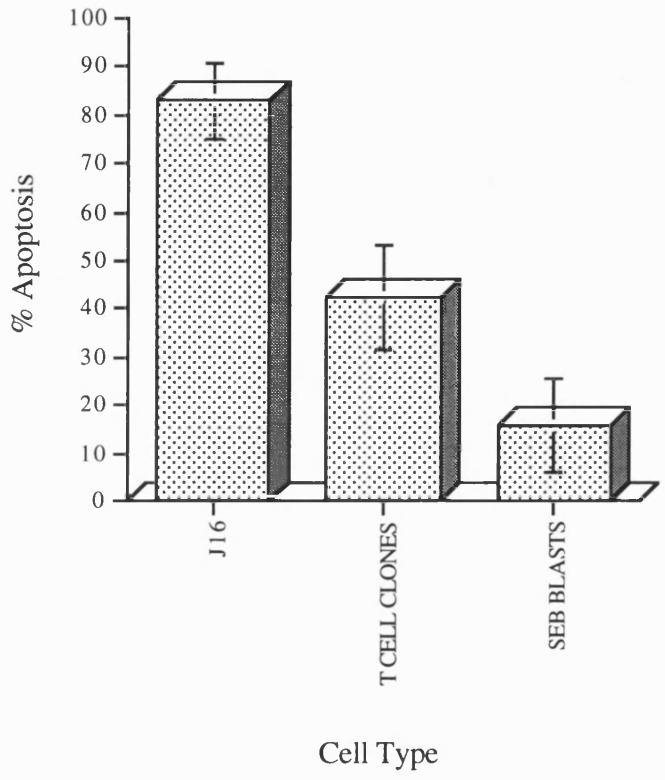


Figure 4.22: Sensitivity to CD95-mediated apoptosis in T cells. J16 cells, 7148 T cell clones (day 6 post stimulation) or SEB blasts (day 6) were incubated for 15h with 0.5µg/ml of the anti-CD95 antibody CH11 (0.05µg/ml for J16 cells) and apoptosis was measured by TUNEL analysis. The % increase in fluorescence relative to control-treated cells is shown. Data are pooled from 4 independent experiments and columns indicate the mean (+/-SEM).

illustrated in figure 4.20 (panel B). Surface staining and FACS analysis of these cells indicated expression of the CD95 receptor (figure 4.21, panel A) and sensitivity to CD95 ligation was therefore tested. Following CH11 treatment, approximately 50% of these cells underwent apoptosis as assessed by TUNEL analysis (figure 4.21, panel B). This analysis indicated that the CD95 pathway was operative in this alloreactive T cell line. In addition, two other T cell clones were analysed in the laboratory and exhibited similar sensitivity to CD95-mediated apoptosis.

In terms of the percentage apoptosis following CD95 challenge, the T cell models investigated could be ranked in order of CD95 sensitivity as indicated in figure 4.22. This figure illustrates the average CD95-induced kill (as measured by TUNEL) from 4 experiments with each of these T cell types and demonstrates clearly that Jurkat cells were most sensitive to CD95 ligation followed by T cell clones and finally normal activated T cells. Since in terms of closeness to a physiological model of an activated T cell, SEB blasts are arguably closest whilst Jurkat cells are furthest removed from normality, it would appear that resistance to apoptosis is a feature of normal T cells which is diminished in T cell clones, and may be absent from transformed T cell lines.

4.3 DISCUSSION

In order to carry out an investigation into the control of programmed cell death, the establishment of a series of assays was performed, to allow apoptosis assessment according to a number of different criteria. This was important since apoptosis is a morphologically defined phenomenon and there is no single criterion which serves universally as a marker for this process. The most widely accepted marker, which is associated with the induction of apoptosis in nearly all systems tested, is the systematic cleavage of nuclear DNA initially yielding

300Kbp and 50Kbp fragments followed subsequently by the appearance of smaller fragments comprising multimers of 180pb (McConkey et al., 1994). Early work on apoptosis relied heavily on the visualisation of DNA fragmentation by the use of laddering assays (Owen-Schaub et al., 1992; Groux et al., 1993), but with the rapid growth of interest in this field came more advanced methodologies which facilitated more accurate quantification of DNA fragmentation. For example the TUNEL assay uses fluorescence-tagged nucleotides to label the free DNA ends created as a result of the fragmentation process and, when monitored by FACS analysis, provides a powerful tool for assessing the extent of apoptosis within a cell population. Alternatively, the JAM assay (Matzinger, 1991) offers a rapid and simple approach to apoptosis detection with the filtration of DNA from lysed cells through glass fibre filter mats serving as an indication of DNA fragmentation. The laddering data presented in figure 4.1(B) supported the general consensus that, whilst a qualitative distinction between viable (control-treated) and apoptotic (CH11-treated) samples was unequivocal, quantitative interpretation of the extent of apoptosis between samples was more difficult. For the purpose of this study, therefore, alternative methods for the assessment of apoptosis were utilised.

A significant advance in apoptosis detection which occurred during the course of this investigation was the development of the annexin-FITC binding assay. By focusing on early changes in membrane phospholipid asymmetry associated with the induction of apoptotic signalling, this assay provided for the first time an early marker of commitment to cell death. As demonstrated in figure 4.4, externalisation of membrane PS (which is bound by annexin-FITC) preceded detectable DNA fragmentation by a substantial time period and thus enabled early apoptotic cells to be discerned with greater sensitivity at early time points. Combined with the use of PI as a marker of cell membrane integrity, this assay provided multiparameter analysis of apoptosis-associated membrane changes,

thereby allowing viable, early apoptotic and late apoptotic populations to be readily distinguished.

The opening experiments (figures 4.1-4.6) therefore represent background work performed to accumulate the necessary methodology and expertise for an investigation into apoptosis of human T cells. In addition, the production of further tools to address this aim is described. For example, a soluble source of CD95L was generated in order that CD95 ligation could be achieved in a physiologically relevant manner as a comparison with antibody (CH11) -driven studies. Interestingly, several differences between ligand and antibody-mediated effects have recently been demonstrated (Suda et al., 1996; Zipp et al., 1997) although no obvious differences were observed during the course of this study. Additionally, a CD95-expressing but CD95-resistant cell line was derived from Jurkat cells by sequential treatment with CH11. This cell line (JLW) proved particularly useful as a control for dissecting the mechanisms of T cell cytotoxicity as measured by the JAM bioassay. The defect in CD95-mediated apoptosis in JLW cells was not targeted for investigation, but interestingly this cell line was found to be sensitive to ceramide-induced apoptosis, exhibiting an equivalent apoptotic response to that of the parent line (J16). One downstream event which has been implicated in CD95 signal transduction is the activation of acidic sphingomyelinase which cleaves sphingomyelin to yield the lipid signalling molecule ceramide. Since the addition of cell soluble ceramide to bypass this step appeared to overcome the defect in apoptosis in JLW cells, this suggests that the coupling of CD95 ligation to acidic sphingomyelinase activation may be defective in this cell line, but that the downstream components of the apoptotic pathway are intact. Interestingly, recent literature has highlighted the complex series of protein recruitments to the CD95 receptor which control the initiation of the death signal at a receptor-proximal location (Chinnaiyan et al., 1995; Kischkel et al., 1995; Yang et al., 1997) making these proteins potential targets for the signalling defect in JLW cells.

Following the establishment of assays and reagents, it was then possible to apply these to the investigation of CD95 expression and function in human T cells. As indicated previously, CD95-mediated apoptosis is thought to be a key mechanism for the negative regulation of the T cell immune system, however a detailed understanding of the physiological circumstances under which this process occurs is largely absent from the literature. As the basis for investigations of this nature, the first question to be addressed regarded the expression of the CD95 receptor and the sensitivity of T cells to apoptosis following its ligation with antibody (CH11) or soluble CD95L.

The striking observation from this series of experiments was the marked lack of apoptosis induction in the majority of activated human T cells following CD95 ligation. It has been reported that although resting and early activated T cells undergo limited CD95-mediated apoptosis, in contrast after several days of *in vitro* cultivation sensitivity to this pathway is acquired (Owen-Schaub et al., 1992; Klas et al., 1993). Clearly the presented data refute this hypothesis since even after prolonged periods in culture the T cells examined remained largely insensitive to apoptosis induction by CD95 ligation. As such, these data are somewhat controversial and therefore warranted detailed scrutiny in an attempt to identify the underlying source of these conflicting conclusions. Obvious sources of experimental variation included firstly differences in the activation stimuli utilised for the expansion of T cells in culture, and secondly the choice of assay methodology employed to assess the extent of apoptosis induction. Accordingly, these issues were addressed by the comparative analysis of T cells activated by a range of different stimuli whilst analysis of apoptosis was undertaken by JAM, TUNEL and annexin-FITC binding to minimise the possibility of artefact at the level of apoptosis measurement. Further controls included the use of soluble CD95L to preclude problems arising from the use of antibody (CH11) for the purpose of CD95 ligation (fig 4.10), and the parallel treatment of Jurkat T cells

indicating that T cell CD95-mediated apoptosis could be both successfully induced and adequately detected under these experimental conditions (figures 4.7, 4.8).

Overall these experiments represented a comprehensive effort to test the initial hypothesis, namely that activated human T cells were largely resistant to CD95-mediated apoptosis even after several days in culture. This premise was tested by a wide range of approaches and nevertheless remained robust, representing the most logical interpretation of these accumulated data. Whilst this concept appears controversial, it is becoming increasingly evident from the literature that a wide range of physiological mechanisms exist for the negative regulation of CD95 signalling, including the recently identified FLIP molecules (Irmler et al., 1997; Thome et al., 1997) and mutant caspase 8 proteins (Boldin et al., 1996; Vincenz and Dixit, 1997), supporting the concept that CD95-mediated apoptosis can be regulated downstream of receptor ligation. In addition, the fact that both double positive (expressing both CD4 and CD8 coreceptors) and single positive thymocytes express equivalent CD95 yet only the former apoptose following CD95 ligation (Ogasawara et al., 1995) provides further evidence that CD95 expression does not necessarily equate to CD95 sensitivity. Thus my data clearly suggest that normal activated human T cells are in fact mainly resistant to CD95-mediated apoptosis.

Since the resistance of activated human T cells to CD95-mediated apoptosis appeared to be a consistent finding, a number of experiments were carried out in an attempt to identify whether such resistance was merely a feature of the T cell culture conditions. One possibility was that the serum present in culture medium was contributing towards the insensitivity to apoptosis since serum deprivation has been reported to promote apoptosis induction (Kulkarni and Mcculloch, 1994; Allen et al., 1995; Lindenboim et al., 1995) and is permissive of *c-myc* -induced apoptosis (Evan et al., 1992). Therefore to address this issue, serum free medium

(AIM-V) was utilised. As illustrated in figure 4.13, this medium was competent to support productive T cell activation and proliferation, as assessed by ^{3}H-thymidine incorporation and surface marker changes. However, cells cultured in this manner did not exhibit an increased apoptotic response following CD95 ligation when compared to cells cultured under routine conditions, rendering it unlikely that growth in serum is responsible for the observed resistance to apoptosis.

Since it has recently been shown that splice variants of the CD95 receptor in lymphocytes can yield truncated CD95 proteins which are soluble (Hughes and Crispe, 1995; Papoff et al., 1996) it was possible that local protection from apoptosis could be afforded by such a mechanism. Interestingly, it has been suggested that levels of soluble CD95 are elevated in SLE patients consistent with a possible defect in CD95-mediated apoptosis associated with this condition (Cheng et al., 1994). In addition, the same study demonstrated that injection of mice with soluble CD95 protein triggered the onset of autoimmune features, presumably reflecting the disruption of CD95-based peripheral tolerance mechanisms. Whilst the inhibition of T cell apoptosis by the production of soluble CD95 proteins has therefore been documented, the CD95 resistance in SEB blasts was not transferable by cell supernatants in this study (figure 4.15) rendering involvement of a soluble factor unlikely. Furthermore, if resistance was due to competitive inhibition by soluble CD95 molecules, this would be expected to be overcome at high concentrations of CH11/CD95L, a prediction which was not borne out by the data.

The examination of cell cycle status in conjunction with apoptosis in SEB blasts (figures 4.16 and 4.17) revealed that S-phase entry was not a requirement for the acquisition of CD95 sensitivity, in fact on the contrary there was preferential apoptosis of cells in the G0/G1 region of the cell cycle histogram. This finding is consistent with recent work in which T cells arrested in G0/G1 were not less

sensitive to CD95-mediated apoptosis but rather exhibited a slightly enhanced apoptotic response (Fournel et al., 1996). The SEB blast analysis contrasts with the situation in J16 cells where approximately equivalent apoptosis was observed between G0/G1 cells and S/G2 cells following CD95 ligation, illustrating the lack of constraint on apoptosis induction regardless of cell cycle status in this cell line.

The fact that CD95-mediated apoptosis was routinely restricted to a subpopulation of T cells in this study and in work by others (Miyawaki et al., 1992; Klas et al., 1993; Alderson et al., 1995) raised the issue of heterogeneity within T cell cultures and therefore a critical question relating to this investigation was how the subset of CD95-sensitive cells differed from the remaining population. One possibility was that Th phenotype could contribute to relative sensitivity to CD95 ligation thus prompting the experiments presented in figures 4.18 and 4.19. Since the starting T cell populations are CD8-depleted for this study, it can be concluded that CD95 resistance in SEB blast lines (which are predominantly but not exclusively CD4+) is not CD8+ T cell dependent as it is also observed in lines generated in the absence of CD8+ cells. However, differential CD95 sensitivity was not detected between cells activated in a Th1-like way (in the presence of IL-12) versus those stimulated such that Th2 differentiation would be favoured (in the presence of IL-4) (figure 4.19, B). The lack of a marker to successfully identify the IL-4 treated cells as Th2-like precludes formal conclusions regarding the influence of Th status on CD95 resistance. However, the evidence that cytokine output is differentially regulated between the IL-12- and IL-4-treated populations provides an indication that these cultures may indeed represent 2 pathways of differentiation. The categorisation of CD4+ T cells has historically been fraught with controversy (Bass et al., 1989; Fiorentino et al., 1989; Mosmann and Coffman, 1989; Karulin and Lehmann, 1997) and a recent cautionary review (Allen and Maizels, 1997) has highlighted the danger of accepting these subdivisions as dogma. One interesting issue raised in this article is the dearth of adequate markers to equivocally distinguish between T helper subsets cells. In the

light of this limitation, it was therefore decided that further experimentation following this approach was not appropriate to this study since it is clear that categorisation of Th cells is a more complex issue than initial reports may have suggested.

The evidence from work on alloreactive T cell clones provides a useful comparison for normal peripheral blood T cells. The rationale underlying this set of experiments was drawn from the fact that much of the published data addressing sensitivity to CD95-mediated apoptosis was based on studies using longer term T cell cultures such as clones or hybridomas (Brunner et al., 1995; Ju et al., 1995; Yang et al., 1995). Such cultures provide an alternative model of activated T cells which represents a useful intermediate between immortalised cell lines and freshly isolated peripheral blood T cells. The fact that apoptosis following CD95 ligation was readily induced in about 50% of the population in these studies (figure 4.21) was encouraging from the perspective that it provided yet another control for the resistance observed in normal activated T cells. Thus there was no universal defect in the ability to induce CD95-mediated apoptosis in human T cells under these experimental conditions. It is of note that the ranking of cell types according to CD95 sensitivity is in accordance with their broad closeness to physiological relevance. Jurkat cells, which represented the greatest divergence from physiology in terms of loss of proliferative controls and immortalisation, exhibited the greatest sensitivity to CD95 ligation whilst freshly isolated activated peripheral blood T cells were the most tightly modulated in this regard. Clones ranked as intermediate, and since they by definition had been subjected to repeated stimulation, and had therefore undergone many population doublings, this raised the intriguing possibility that sensitivity to CD95-mediated apoptosis might be a function of approximation to cellular senescence. In support, it has been recently observed that ageing of human T cell clones is associated with an increase in the sensitivity to apoptosis via this route (Pawelec et al., 1996).

The differential sensitivity of Jurkats, clones and normal activated T cells to apoptosis is enlightening and prompted a closer analysis of the cell types used to form the prevailing hypothesis that activated T cells are CD95 sensitive. The majority of studies linking CD95 ligation with an apoptotic outcome utilised immortalised cell lines such as Jurkats, CEM cells and HELA cells (Yonehara et al., 1989; Ogasawara et al., 1993; Suda et al., 1994; Suda and Nagata, 1994) and the remaining reports have largely been confined to murine hybridomas (Brunner et al., 1995; Ju et al., 1995) or T cell clones (Ramsdell et al., 1994a; Hargreaves et al., 1997). Given the differential sensitivity of cloned *versus* normal activated T cells observed in this study, it is clear that the extrapolation of findings between T cell models is potentially misleading and equally the possibility of species-specific differences between murine and human T cells cannot be ruled out. Thus the only reports which can be considered to contradict the data presented in this study are those pertaining to CD95 sensitivity in human activated peripheral blood T cells. In this regard, activated human T cells were initially reported to be resistant to CD95-mediated apoptosis (Miyawaki et al., 1992) but two subsequent reports claimed that sensitivity to apoptosis via this route was acquired after four (Owen-Schaub et al., 1992) or six (Klas et al., 1993) days of *in vitro* activation, contrasting with the data presented in this thesis. Closer analysis of the study by Klas and colleagues reveals that sensitivity to apoptosis was construed from CD95-specific increases in TUNEL positivity of 51%, 57.6% and 59.6% indicating that a proportion of the population nevertheless exhibited resistance to CD95 ligation. Another important difference between these reports and the data presented in this study relates to the T cell activation protocols employed and the relevance of this will become apparent in the light of experiments detailed in chapter 6.

In summary, these data demonstrated that normal activated peripheral blood T cells exhibited a restricted apoptotic response following CD95 engagement, and that this could not be attributed to the type of activation stimulus employed, the

nature of ligand provision (antibody *versus* natural ligand) nor was it a function of the criteria used to assess apoptosis induction. Resistance was observed in SEB blasts generated from purified CD4+ T cells, it was not serum dependent and was not transferable by a soluble factor. Cells from all phases of the cell cycle exhibited CD95 resistance, although this was particularly marked in the S/G2 portion of the DNA histogram. An investigation of the potential mechanisms underlying control of CD95 resistance is presented in chapter 6 of this thesis.

CHAPTER 5

CD95-Ligand Expression in T Cells: Relevance to Activation induced Cell Death (AICD)

5.1 INTRODUCTION

Given the inherent danger in the existence of biological pathways which trigger cell death, it is likely that such pathways are subject to regulation at a number of levels, and controlling sensitivity to apoptosis following CD95 ligation is one potential mechanism of achieving this. A further obvious point of control is the provision of ligand for these death-inducing receptors, and accordingly, cellular distribution of CD95L is tightly restricted, with expression being mainly limited to low levels on activated T lymphocytes (Suda et al., 1994; Suda and Nagata, 1994) as well as on some APCs (Badley et al., 1997; Lu et al., 1997a).

Since T cells upregulate the CD95 receptor upon activation (as illustrated in the previous chapter), it is apparent that the potential for cell suicide exists under these circumstances and indeed this forms the basis for the concept of activation induced cell death (AICD). Thus, physiological regulation of the CD95 system in T cells may occur in part by the upregulation of CD95L following TCR ligation during the activation process. Signalling via the TCRζ ITAM has been shown to be sufficient to mediate CD95L upregulation (Vignaux et al., 1995), and the process is believed to be calcium-dependent (Anel et al., 1994; Vignaux et al., 1995). Thus whilst antigen engagement can result in positive outcomes such as the initiation of proliferation, conversely under certain circumstances antigen-mediated signals can negatively regulate T cell responses, and may even trigger the induction of apoptotic pathways.

The following experiments examined the ability of activated T blasts as compared to resting cells to express CD95L. In addition, the effect of TCR restimulation on CD95L expression in previously activated T cells was examined.

5.2 RESULTS

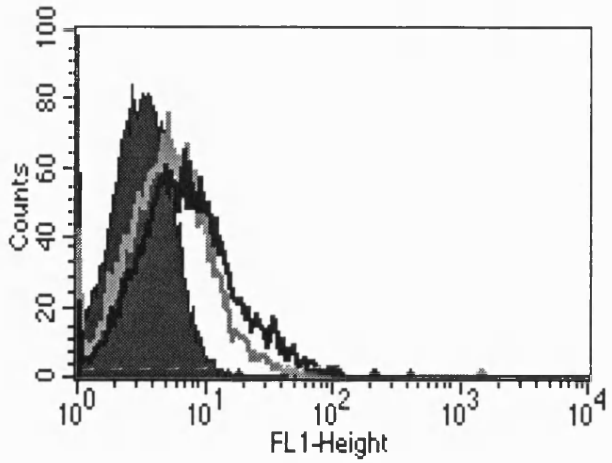
5.2.1 FACS Staining for CD95L

In order to investigate the possible expression of CD95L on human T cells, surface staining and FACS analysis was utilised. Early experiments failed to detect CD95L staining, and during the course of these studies it emerged that surface CD95L is subject to metalloproteinase cleavage (Kayagaki et al., 1995; Mariani et al., 1995) to allow release as a soluble factor. Therefore detection by FACS staining required the use of a metalloproteinase inhibitor (BB2116). Fig 5.1 (A) illustrates that under conditions of P/I stimulation, in the presence of such an inhibitor, CD95L could be visualised at low levels. The specificity of the anti-NOK1 antibody was demonstrated by the inhibition of soluble CD95L-induced apoptosis (figure 5.1, panel B) yet the detection of surface CD95L using this antibody was nevertheless marginal. Since the expression levels detected under optimal staining conditions were considered too marginal to be routinely useful, a bioassay based on the ability of T cells to kill CD95-positive ^{3}H-thymidine labelled Jurkats in the JAM assay was used for assessment of CD95L expression in this study.

5.2.2 Activation-induced Cytotoxicity in T Cells

An early observation emanating from these studies was that activated but not resting T cells were able to induce apoptosis in a target population comprising ^{3}H-thymidine labelled J16 cells (figure 5.2, panel A). Accordingly, the addition of resting PBMC had little effect on the viability of the Jurkat population, whilst PBMC which had been cultured in the presence of SEB for 4 days were competent to induce apoptosis in this bioassay. The extent of apoptosis induction exhibited

(A)



(B)

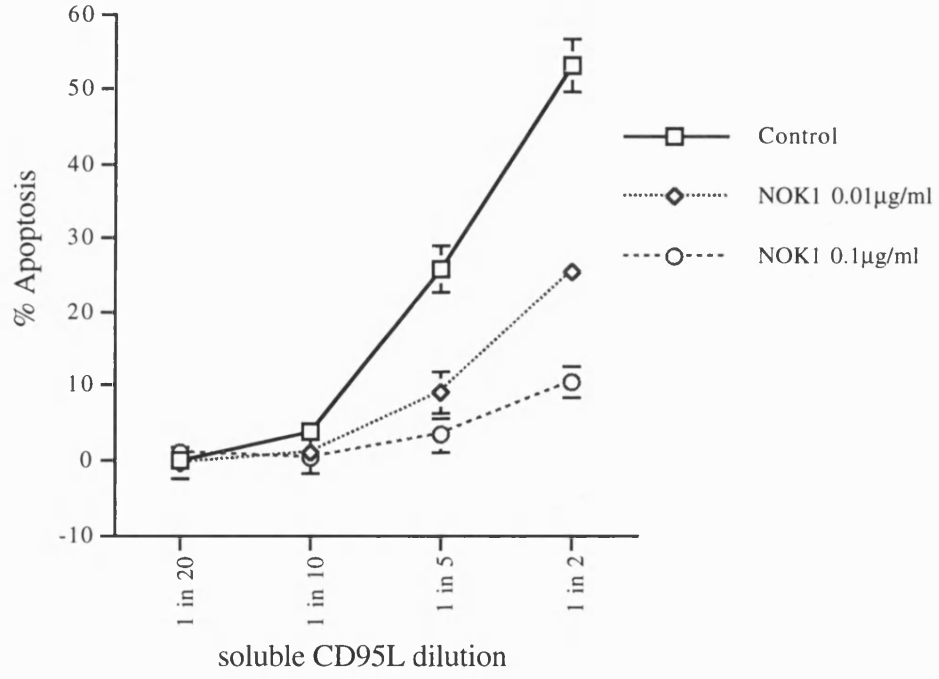


Figure 5.1: Expression of CD95L on activated T cells. (A) Day 6 SEB blasts were activated for 6h with PMA (0.04µg/ml) and ionomycin (1µM) in the presence (open histograms) or absence (filled histogram) of the metalloproteinase inhibitor BB2116 (Grey line = 2µg/ml, black line = 5µg/ml). Staining was performed using the anti-CD95L antibody NOK1 (1µg/ml) followed by anti-mouse polyvalent-FITC. Data indicate the maximum staining observed in 5 experiments. (B) To demonstrate CD95L binding, NOK1 was used to inhibit soluble CD95L-induced apoptosis of ^{3}H-thymidine-labelled J16 cells in an 8h JAM assay. Data are representative of 3 experiments.

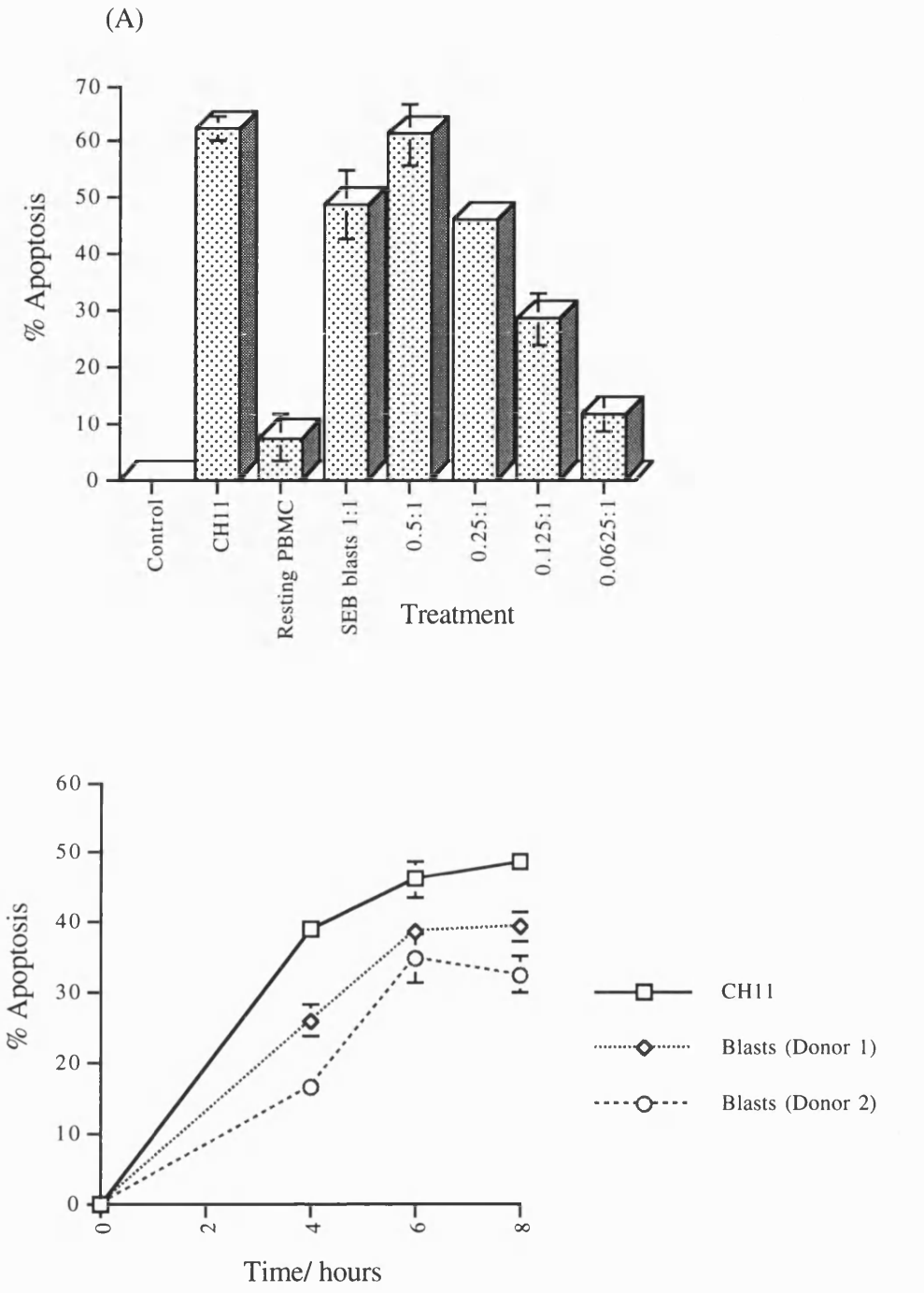


Figure 5.2: Activation-induced cytotoxicity in T cells. (A) Day 3 SEB blasts (or resting PBMC) were incubated for 15h with ^{3}H-thymidine-labelled J16 cells at the indicated ratios (effector:target). (B) SEB blasts (day 3) were incubated with ^{3}H-thymidine-labelled J16 cells (1:1 ratio) for the indicated time period. The anti-CD95 antibody CH11 was used at 0.05µg/ml. Apoptosis was measured by JAM assay and is calculated as the % decrease in CPM relative to control-treated cells. Data indicate the mean (+/-SEM) of triplicate wells and are representative of 4 similar experiments.

dose dependence and it is notable that even low ratios of effector cells were sufficient to induce apoptosis under these conditions, demonstrating the potency of this response. These ratios are considerably lower than those used in standard perforin/granzyme CTL assays, and furthermore the lack of a T cell receptor target on Jurkat T cells indicates that cytotoxicity is not dependent on allorecognition. In addition, the potency of the response even at low ratios of effector:target cells indicated the possible involvement of a soluble mediator rather than a requirement for cell:cell contact. Inclusion of CH11 in this assay served as a positive control for apoptosis induction and interestingly triggered broadly equivalent cytotoxicity to that induced by the higher ratios of SEB blasts.

To further examine the cytotoxicity exhibited by activated T cells, kinetic analysis was performed. These data (figure 5.2, panel B) revealed that co-incubation of SEB blasts with ^{3}H-thymidine labelled Jurkat cells for as little as 3 hours resulted in detectable apoptosis of target cells and that this effect was maximal by 6 hours. PBMC from two donors were used in this experiment and were cultured for 4 days with SEB prior to use in the JAM bioassay. The kinetics of apoptosis induction by these two SEB blast cultures were similar, and interestingly closely mimicked that induced by the anti-CD95 antibody CH11. In contrast, apoptosis induced by perforin and granzymes is known to occur with substantially slower kinetics (D. Sansom, personal communication).

To determine the duration of this ability to induce apoptosis, SEB blasts were tested on a daily basis following initial activation with SEB, revealing that even up to 6 days post stimulation, the cells were competent to kill J16 cells (figure 5.3, panel A). In contrast, resting PBMC which remained in medium in the absence of SEB for this time period, were unable to induce apoptosis in the labelled J16 cells, indicating that the mechanism(s) underlying this process were induced by T cell activation. Acquisition of this cytotoxic potential in peripheral blood T cells was not a specific feature of superantigen addition, since the activation of PBMC with

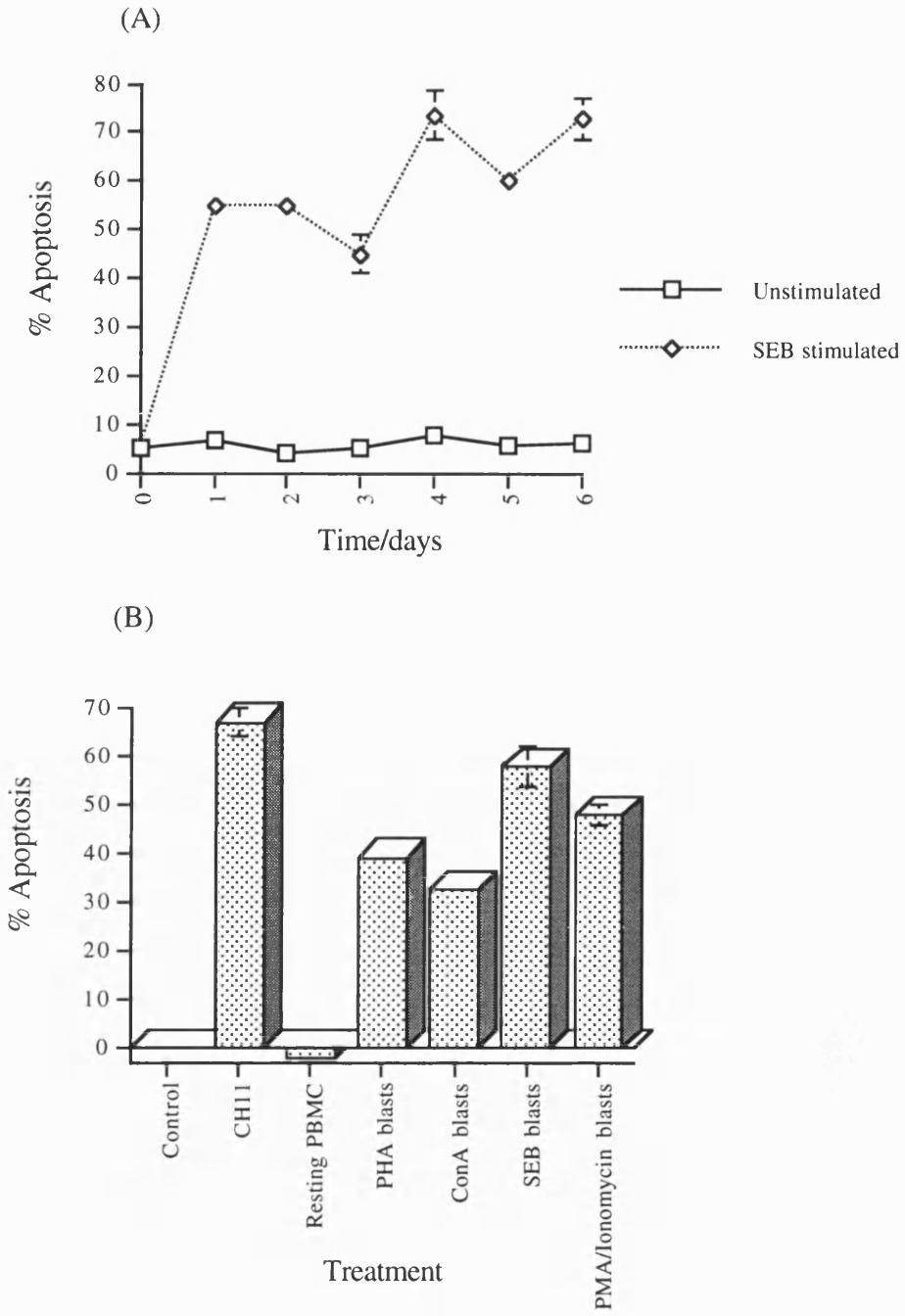


Figure 5.3: Duration of activation-induced cytotoxicity in T cells. (A) SEB blasts on the indicated day post stimulation were incubated for 15h with ^{3}H-thymidine-labelled J16 cells (1:1 ratio). Apoptosis was measured by JAM assay and is plotted as the % decrease in CPM relative to control-treated cells. Data are representative of 2 experiments. (B) Resting PBMC were activated for 3 days with PHA (2µg/ml), ConA (1µg/ml), SEB (1µg/ml) or a combination of PMA (0.04µg/ml) and ionomycin (1µM). The % apoptosis (by JAM assay) relative to control-treated cells is shown. Data indicate the mean (+/-SEM) of triplicate wells and are representative of 3 similar experiments.

alternative stimuli including PHA, ConA, or a combination of PMA and ionomycin also triggered this process (figure 5.3, panel B). These data therefore indicated that T cell activation, by diverse stimuli, induced a mechanism(s) of cytotoxicity which could be visualised by the rapid induction of apoptotic cell death in CD95 positive J16 cells, the kinetics and potency of which were consistent with a potential role for the CD95 pathway.

In order to examine the contribution of CD95L upregulation to the ability of SEB blasts to induce apoptosis, CD95-specific blocking reagents were employed. Resting purified T cells were therefore activated with SEB (presented on HLA-DR/CD80 transfectants) in the presence of the anti-CD95 blocking antibody M3 and cytotoxicity was measured by co-incubation with ^{3}H-thymidine labelled J16 cells. As in previous experiments, addition of CH11 to the J16 cells served as a positive control for the JAM bioassay. Consistent with the data presented in figures 5.2 and 5.3, figure 5.4 illustrates the requirement for cellular activation for the induction of cytotoxic potential since resting T cells or those treated with HLA-DR/CD80 transfectants in the absence of SEB failed to induce apoptosis in the Jurkat population. In addition, this experiment demonstrated that the mechanism of cytotoxicity was CD95-dependent, since inclusion of the anti-CD95 blocking antibody M3 ablated the induction of apoptosis by activated T cells (figure 5.4). With the exception of M3, which was pre-incubated with the target cells (J16) for one hour, there was simultaneous addition of all cells and reagents in this experiment, such that both the induction and execution of cytotoxicity occurred during the course of this 15 hour assay. Since the observed apoptosis was CD95-mediated, this indicated that CD95L upregulation was an early event during human T cell activation and was functional less than 15 hours following addition of the activation stimulus.

At this early time point, therefore, activation-induced cytotoxicity appeared to be almost entirely attributable to CD95L expression, since it was effectively blocked

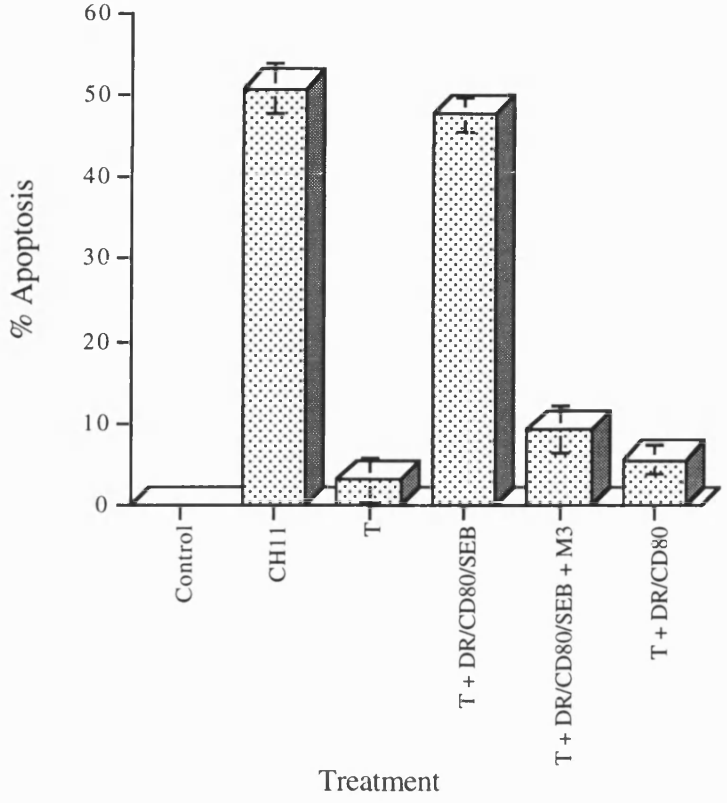


Figure 5.4: Inhibition of activation-induced T cell cytotoxicity by anti-CD95 blocking antibody. Resting purified T cells were treated with SEB-pulsed/unpulsed transfectants as indicated in the presence of ^{3}H-thymidine-labelled J16 cells (1:1 ratio). Anti-CD95 blocking antibody (M3) was used at 3µg/ml and was pre-incubated with J16 cells for 1h. The apoptotic anti-CD95 antibody CH11 was used at 0.05µg/ml. Apoptosis of J16 cells (calculated relative to control-treated cells) was assessed by JAM assay and data are representative of 3 independent experiments.

by specific inhibitors of the CD95 pathway. However at later time points following activation there was more variability in the ability of CD95-specific reagents to block the induction of apoptosis by SEB blasts, indicating that pathways other than CD95 may be utilised under some circumstances. For example, in figure 5.5 (panel A), the SEB blast line derived from donor 1 induced apoptosis by a mechanism which was clearly dependent on CD95L expression, since it was blocked by CD95-Fc. In contrast, the SEB blast culture derived from donor 2 exhibited broadly equivalent apoptosis induction to the donor 1-derived line in terms of the percentage cytotoxicity, yet showed no decrease in cytotoxicity following the inclusion of the CD95-specific blocking reagent CD95-Fc, indicative of a non CD95-dependent mechanism.

To verify that this result was not a feature of the limitations of the blocking reagents utilised, these SEB blasts were also tested for cytotoxicity against ^{3}H-thymidine labelled JLW cells in parallel with J16 cells. Since JLW cells had been selected for resistance to apoptosis via the CD95 pathway (Chapter 4.2.2), they provided a useful tool for the detection of apoptosis-inducing interactions which did not occur via this route. Interestingly, SEB blasts which killed J16 cells in a manner which was not inhibited by CD95-Fc were equally potent in their ability to kill JLW cells, whilst SEB blasts which failed to kill in the presence of CD95-Fc were unable to induce apoptosis in JLW cells (figure 5.5, panel B). These data therefore indicated the existence of alternative inducible cytotoxic ligands on T cells in addition to CD95L. At the time of these studies, a major candidate for such a pathway was TNFα which was known to be expressed in T cells under various circumstances. However since Jurkat T cells are resistant to TNF induced apoptosis (Wong and Goeddel, 1994), the killing detected in the JAM bioassays was unlikely to have occurred via this route suggesting the existence of still more death pathways. In this regard, the recent discovery of several new members of the CD95/TNFR family and associated T cell ligands (Chinnaiyan et al., 1996;

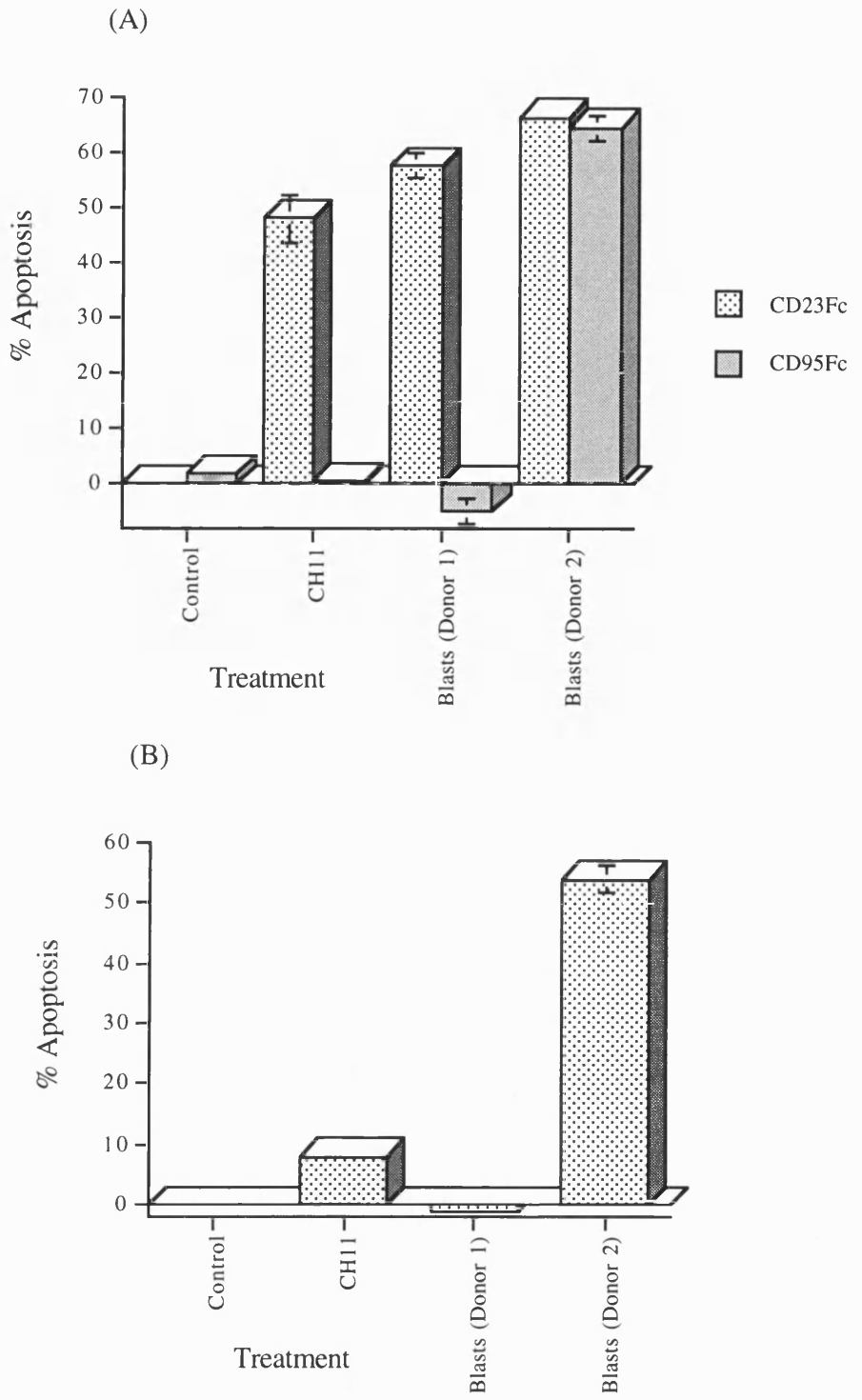


Figure 5.5: Effect of CD95-Fc on cytotoxicity of T blasts. SEB blasts (day 6) were incubated for 6h with ^{3}H-thymidine-labelled J16 cells (A) or ^{3}H-thymidine-labelled JLW cells (B) (1:1 ratio) in the presence of CD95-Fc or a control Fc construct (CD23-Fc). Anti-CD95 antibody (CH11) was used at 0.1µg/ml. Apoptosis was measured by JAM assay and the % decrease in CPM relative to control-treated cells is shown. Columns show the mean (+/-SEM) or triplicate wells. Data are representative of 4 similar experiments.

Pan et al., 1997) makes these pathways potential candidates for the CD95-independent mechanism(s) of cytotoxicity utilised by activated SEB blasts.

5.2.3 TCR-induced CD95L Upregulation in Activated T Cells

The initial TCR-mediated activation of resting T cells is considered unlikely to mediate AICD, since resting T cells require time for CD95 upregulation and acquisition of CD95 sensitivity before undergoing apoptosis (Wesselborg et al., 1993). However, following activation, CD95 expression remains high in T cells, and restimulation via the TCR can therefore potentially trigger apoptosis induction (Wesselborg et al., 1993). In order to investigate TCR restimulation in previously activated T cells, TCR signalling was provided by the use of superantigen or anti-CD3 antibody. Thus in these experiments, previously activated T cell blasts were restimulated and apoptosis was measured.

In order to test the ability of SEB-mediated TCR restimulation to induce apoptosis in activated T cells, SEB blasts were ^{3}H-thymidine labelled and exposed to a range of concentrations of SEB to assess the induction of AICD in the JAM assay. Thus in contrast to previous assays, the viability of the SEB blasts themselves (rather than a target J16 population) was assessed and the aim was to study the effect of TCR restimulation in cells which had already once been activated with superantigen. For these experiments, SEB was added directly to culture wells rather than being pulsed onto HLA-DR/CD80 transfectants since we have previously shown that the provision of APCs upon SEB rechallenge of T blasts favours a proliferative rather than an apoptotic response (Boshell et al., 1996). As illustrated in figure 5.6, whilst a degree of apoptosis was detectable in response to high dose SEB addition (donor 1), the proportion of sensitive cells was low, and in all experiments of this nature performed (>6) the amount of apoptosis observed did not exceed 30%. Indeed, many SEB blast lines appeared to be strikingly

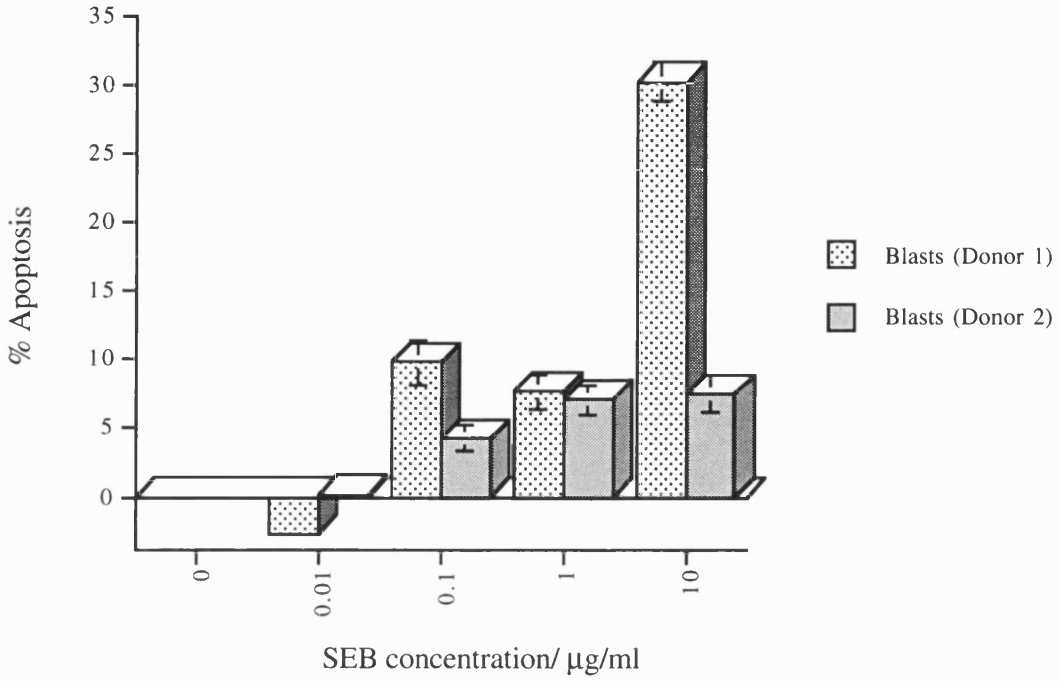


Figure 5.6: Effect of TCR restimulation using SEB on apoptosis of T blasts. Day 6 ^{3}H-thymidine-labelled SEB blasts were incubated for 15h with the indicated concentrations of SEB and apoptosis was measured by JAM and is calculated as % decrease in CPM relative to control-treated cells. Columns show the mean (+/-SEM) of triplicate wells and the experiment was repeated with 4 separate donors with similar results.

resistant to the induction of AICD via SEB treatment as reflected by the response of cells from donor 2 in this experiment.

One interpretation of these results was that SEB stimulation of previously activated T blasts failed to induce sufficient TCR signalling to allow CD95L upregulation, although this was clearly not the case for resting T cells activated with HLA-DR/CD80 presented SEB as demonstrated earlier (figure 5.4). To address this possibility, the induction of CD95L during restimulation of T blasts with SEB was examined using ^{3}H-thymidine labelled J16 cells as targets. Figure 5.7 illustrates that whilst the basal apoptosis induced by SEB blasts could be CD95-independent (consistent with previous findings), when blasts were restimulated with SEB they became more effective at killing J16 cells, and furthermore this inducible cytotoxicity was largely CD95-dependent. Accordingly, the additional apoptosis which occurred when T blasts were further stimulated with SEB was markedly inhibited in the presence of the anti-CD95 blocking antibody M3. Thus, stimulation with SEB induced upregulation of functional CD95L in these T cell blasts rendering them competent to induce apoptosis in ^{3}H-thymidine labelled CD95 positive target cells.

Thus it appeared that whilst TCR restimulation with SEB was sufficient to upregulate CD95L expression, the majority of T blasts were nevertheless protected from apoptosis under these circumstances, despite the consequent coexpression of CD95 and CD95L in these cultures. This finding once more highlighted that a number of distinct controls appeared to regulate CD95-mediated apoptosis in normal activated T cells.

An alternative method for mimicking antigen-derived signals is direct ligation of the TCR CD3 component, and therefore investigations were also carried out to assess the competence of anti-CD3 antibody to trigger AICD in T blasts. Since the use of antibody to ligate the TCR CD3 component bypasses TCR specificity,

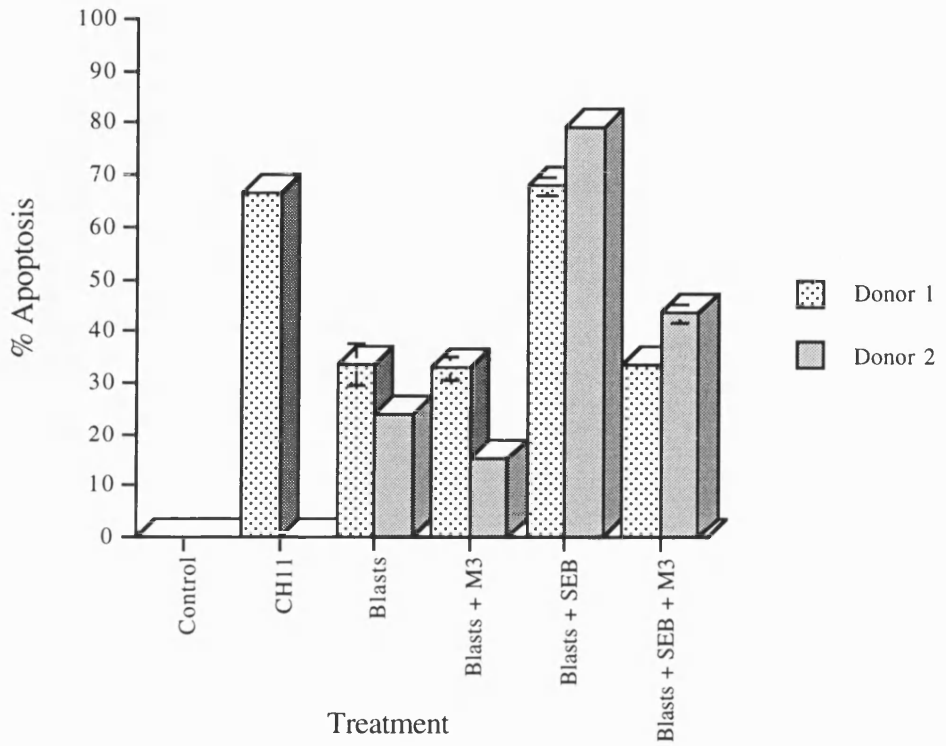


Figure 5.7: Effect of anti-CD95 blocking antibody on cytotoxicity induced by TCR restimulation using SEB. Day 6 SEB blasts were incubated for 15h with ^{3}H-thymidine-labelled J16 cells (1:1 ratio) in the presence of 1µg/ml SEB and/or the anti-CD95 blocking antibody M3 (3µg/ml) where indicated. The apoptotic antibody CH11 was used at 0.05µg/ml. Data are representative of results obtained from 4 donors.

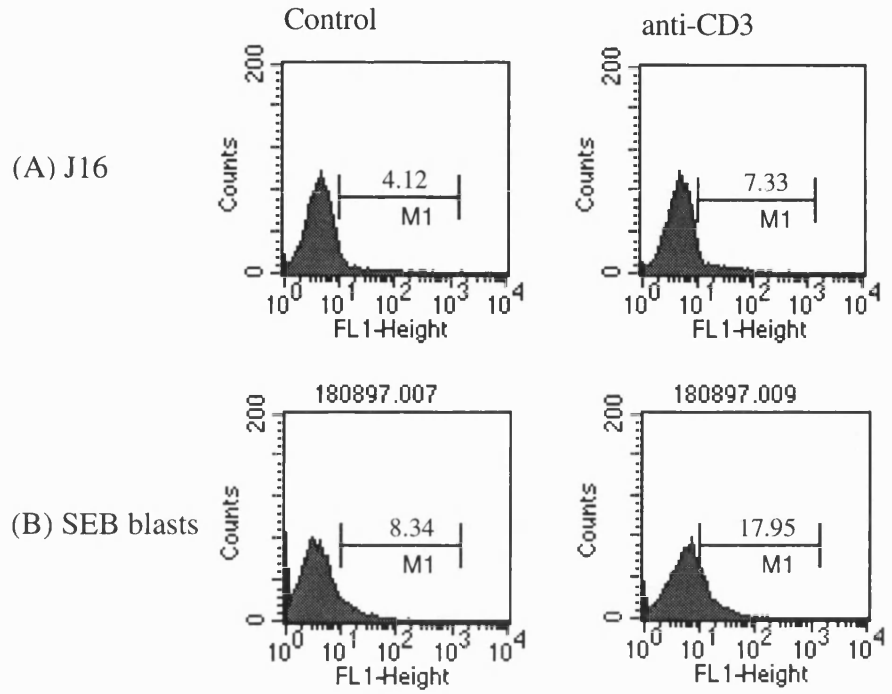


Figure 5.8: Effect of anti-CD3 treatment on apoptosis induction in J16 cells and SEB blasts. J16 cells or day 6 SEB blasts were incubated for 24h on immobilised anti-CD3 (plate-coated for 15h at 10µg/ml) and apoptosis was measured by TUNEL analysis. Anti-CD3 dependent increases in TUNEL fluorescence of 3.21% (J16) and 9.61% (SEB blasts) were observed. Data are representative of >5 similar experiments.

this approach was also applicable to Jurkat T cells. Accordingly, both J16 cells and SEB blasts were subjected to a 15 hour incubation with immobilised anti-CD3 prior to apoptosis measurement using the TUNEL assay. Figure 5.8 illustrates the results of this analysis, which revealed only a 3% increase in TUNEL positivity for J16 cells, whilst in SEB blasts, the extent of apoptosis in response to anti-CD3 was also limited (< 10%). Restimulation of activated T cells using anti-CD3 antibody therefore did not appear to provide an effective trigger for the induction of AICD, suggesting either a lack of CD95L induction or that the cells were not susceptible to CD95-mediated apoptosis under these conditions.

Given the low apoptotic response observed in these experiments, the effectiveness of the anti-CD3 antibody was therefore investigated. A key event in the induction of AICD is the mobilisation of intracellular calcium which has been demonstrated to be a requirement for TCR-mediated CD95L upregulation in studies using cyclosporin A (Vignaux et al., 1995). In order to verify the ability of the anti-CD3 antibody to effectively elevate intracellular calcium ion concentration in activated T cells, assays to detect this process were carried out. Thus the aim of these experiments was to validate the anti-CD3 antibody rather than to provide a quantitative analysis of intracellular calcium levels. Calcium mobilisation was measured by fluo-3 loading of cells and an ionomycin titration was performed to establish the effectiveness of the assay. As illustrated in figure 5.9 (panel A), increasing concentrations of ionomycin resulted in calcium fluxes of similar duration but larger amplitude, as measured by an increase in MFI (mean fluorescence intensity). Similarly, treatment of T blasts with anti-CD3 antibody (OKT3) in conjunction with crosslinker (anti-mouse Ig) triggered dose-dependent calcium fluxes verifying that this antibody was competent to induce calcium mobilisation in these cells. This experiment also demonstrated the requirement for crosslinking of the anti-CD3 antibody (using anti-mouse Ig) for the initiation of calcium signalling as detected in this assay. An alternative method for the crosslinking of antibodies is the process of plate-coating, which was used for the

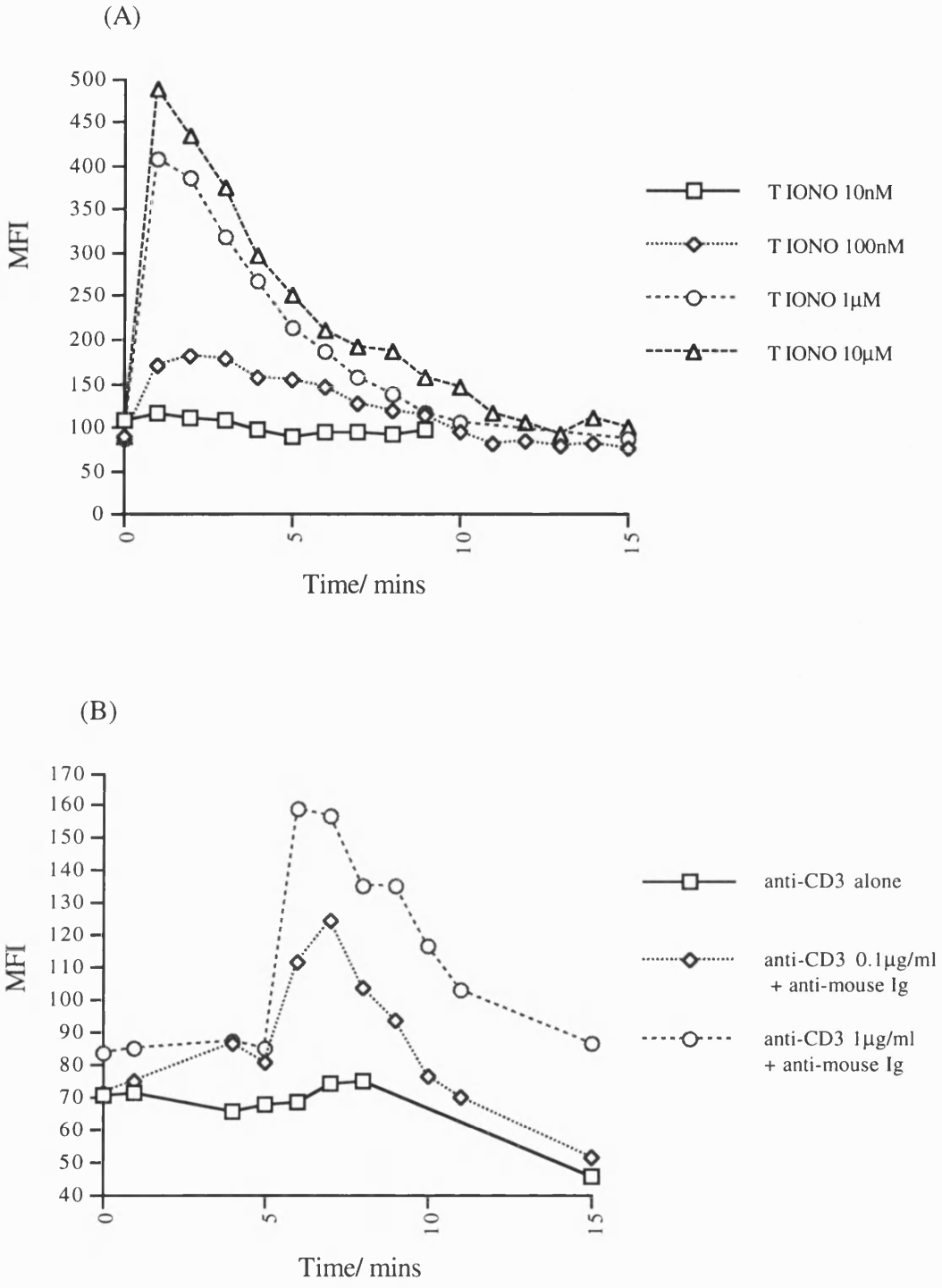


Figure 5.9: Measurement of intracellular calcium mobilisation in T cells. (A) Day 6 SEB blasts were loaded with fluo-3 and treated with the indicated concentrations of ionomycin (IONO). (B) Fluo-3 loaded SEB blasts (day 6) were treated with anti-CD3 antibody (OKT3) at the indicated concentrations and crosslinker (anti-mouse Ig) was added at the 5min time point where indicated. Calcium mobilisation was measured by an increase in FL-1 fluorescence and the mean fluorescence intensity (MFI) is shown. Data are representative of 3 experiments.

anti-CD3 treatment in figure 5.8 as well as for the proliferation assays presented in chapter 3 of this thesis.

Having established that this anti-CD3 antibody was competent to induce calcium mobilisation, the effect of anti-CD3 treatment on CD95L expression was examined in both Jurkat cells and T blasts with the use of the JAM assay (figure 5.10). Panel A of this figure illustrates the viability of ^{3}H-thymidine labelled Jurkats following treatment with immobilised anti-CD3 antibody, and demonstrated a lack of detectable apoptosis induction. This finding suggested that CD95L was not upregulated in J16 cells under these experimental conditions. Contrasting with this result, the incubation of normal activated T blasts with immobilised anti-CD3 indicated upregulation of CD95L in this cell type, since the ability to kill CD95 positive Jurkat cells in the JAM bioassay was induced under these conditions. The specificity of the induced cytotoxicity was shown to be CD95-dependent since it was inhibited in the presence of CD95-Fc but not the control Fc construct (CD23-Fc).

Since there is some evidence that CD28 ligation can promote T cell survival during activation (Groux et al., 1992; Radvanyi et al., 1996) experiments were carried out to determine whether CD80-mediated signals might confer protection from AICD by inhibiting the process of CD95L upregulation. Accordingly, the effect of anti-CD3 in the presence of CHO-CD80 cells was also monitored in terms of the degree of CD95L induction in the JAM bioassay (figure 5.10, panel B). This analysis revealed that costimulatory signals through this route did not appear to affect the extent of CD95L upregulated following TCR ligation, suggesting that if CD80 does indeed protect from AICD, the mechanism would appear to be downstream of CD95L induction.

Overall, these data indicated that both SEB stimulation and anti-CD3 treatment triggered the upregulation of functional CD95L in activated T blasts, yet despite

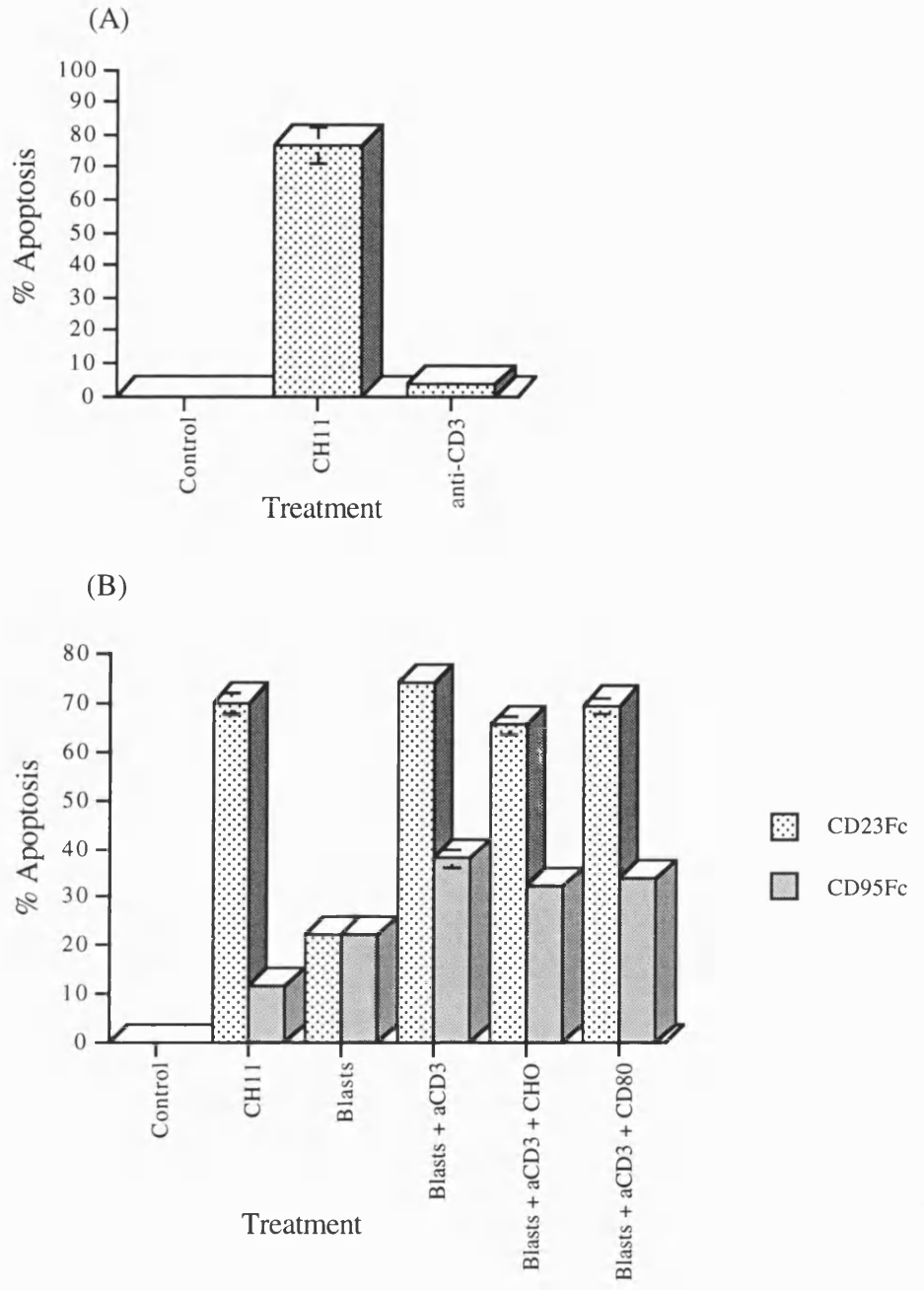


Figure 5.10: Effect of anti-CD3 treatment on apoptosis induction in T cells. (A) ^{3}H-thymidine labelled J16 cells were incubated for 15h with immobilised anti-CD3 (plate-coated at 10µg/ml) and apoptosis was measured by JAM assay. The anti-CD95 antibody CH11 (0.05µg/ml) served as a positive control for apoptosis induction. (B) Day 6 SEB blasts were incubated with anti-CD3 antibody (plate coated at 10µg/ml) and the induction of CD95L was measured by apoptosis induction in target ^{3}H-thymidine labelled J16 cells. CD95-Fc or a control Fc construct (CD23-Fc) were used at a 1 in 5 dilution. CHO transfectants were used at a ratio of 1:5 (transfectant:T cell). Apoptosis was calculated as the % decrease in CPM relative to control-treated cells and the mean (+/-SEM) of triplicate wells is shown. Data are representative of 3 experiments.

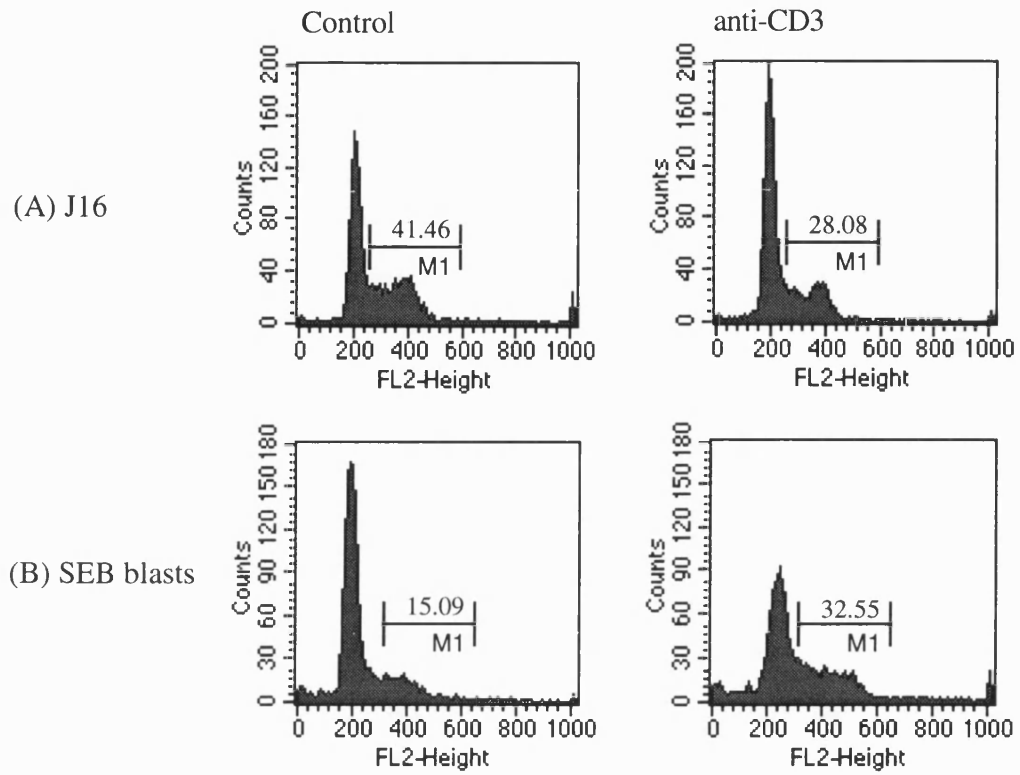


Figure 5.11: Effect of anti-CD3 treatment on cell cycle in T cells. J16 cells (A) or day 6 SEB blasts (B) were exposed to immobilised anti-CD3 (plate-coated at 10µg/ml) for 24h then cell cycle status was analysed by PI staining. Data are representative of 3 individual experiments.

co-expressing both CD95 and its ligand, the T blasts themselves appeared to be spared from apoptosis via this route. In contrast, whilst Jurkat T cells were also resistant to anti-CD3 induced apoptosis under these conditions, this was likely to be due to a failure to effectively upregulate CD95L in this cell type, emphasising the potential for variation between cell types in the control of the CD95 system.

In order to further characterise the response to CD3 ligation in both Jurkats and normal T cells, experiments were undertaken to establish whether this signal had any observable effect on cell cycle, for example by mediated cell cycle arrest since this has been reported for some transformed T cell lines (Zhu and Anasetti, 1995). Consistent with such reports, figure 5.11 demonstrates a decrease in the number of cells in S/G2 phase following anti-CD3 treatment of J16 cells (A) indicative of growth arrest at the G1/S-phase checkpoint. In contrast, the cell cycle trace for SEB blasts (B), showed a marked increase in the number of cells present in the S/G2-phase implying that rather than mediating growth arrest, signalling through the CD3 complex appeared to be favouring S-phase entry in this cell type. This is significant in that it provided further evidence of functionality associated with the use of this anti-CD3 antibody, and yet a clear lack of apoptosis in the presented data suggested that outcomes other than cell death could be initiated under these circumstances.

5.3 DISCUSSION

The experiments performed in this chapter established that activated but not resting human T cells exhibited cytotoxicity against Jurkat cells which proceeded with rapid kinetics reminiscent of that observed for CD95-induced apoptosis. The acquisition of cytotoxic potential occurred irrespective of the initial activation

stimulus and was observed for cells stimulated with PHA, ConA, P/I or SEB. The data further demonstrated that immediately post T cell stimulation with SEB, the induced cytotoxicity was largely attributable to the expression of CD95L since it was inhibited in the presence of CD95-specific blocking reagents. Thus CD95L upregulation was shown to be an early event occurring during the first 15 hours of the T cell activation process (figure 5.4). This is consistent with several reports which have documented upregulation of mRNA for CD95L during the first 3-12 hours following TCR stimulation (Brunner et al., 1995; Ju et al., 1995; Vignaux et al., 1995; Yang et al., 1995).

Therefore as immune responses are initiated and T cells acquire stimulatory functions, the propensity to undergo apoptosis is simultaneously increased via the upregulation of the mediators of cell death (CD95 and CD95L). The coupling of the activation process to elements of an apoptotic pathway may represent an intrinsic control mechanism designed to preclude unregulated immune stimulation which poses a potential threat to the host. Whilst the data presented in the previous chapter indicate that the CD95 death pathway is not constitutively functional in activated T cells, nevertheless the induction of both CD95 and its ligand during the activation process confers the potential to initiate an apoptotic signal under circumstances when CD95 sensitivity is acquired.

An interesting point arising from these experiments is that CD95-dependent cytotoxicity is detectable in certain T blast lines as long as 6 days following the initial activation stimulus (figure 5.5). Published reports on CD95L expression have focused on the transient upregulation of mRNA and protein at early time points (Brunner et al., 1995; Ju et al., 1995) and the persistence of CD95L expression subsequent to this time has not been addressed. In one report, the authors state that activated T cells do not express CD95L prior to TCR restimulation (Vignaux et al., 1995), however the data on which this premise is based are not presented. Since the sensitivity of CD95L surface staining protocols

is low (figure 5.1), and activated T cell lines appear heterogeneous in their capacity to express this protein as judged by functional assays (figure 5.5), it is clear that CD95L expression on activated T cells could potentially be overlooked. The data presented in this thesis provide evidence for functional CD95L expression 6 days following activation in certain T cell lines, and it would be of interest to characterise this finding further. For example, future studies could investigate the kinetics of CD95L mRNA changes relative to protein changes during the 6 days following T cell stimulation to determine whether CD95L expression persists or whether the reported transient upregulation of mRNA and protein (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995) is followed by a second wave of CD95L synthesis.

It is also of note that T blasts examined on day 6 following activation exhibited cytotoxicity which was not necessarily attributable to CD95L expression since CD95-specific blocking reagents failed to universally inhibit apoptosis induction. The expression of CD95-independent cytotoxicity was not a donor-specific phenomenon but rather seemed to reflect the existence of alternative cytotoxic mechanisms in activated human T cells. Clearly alternative pathways of apoptosis induction could be triggered in these cells during SEB stimulation and, whilst their nature is undefined in these studies, it is possible that other death-inducing ligands such as TRAIL or the as yet undefined DR3-ligand (Wiley et al., 1995; Kitson et al., 1996; Chinnaiyan et al., 1996) as well as TNFα or the classical secretory pathway of perforin and granzymes may be involved. Whatever the mechanisms underlying the ability of these T cells to induce the apoptosis of Jurkats, the persistence of some form of cytotoxicity at least during the first 6 days of activation (figure 5.3, A) implied that T cells must maintain an "apoptosis resistant" phenotype in order to survive this time period.

Having examined the acquisition of cytotoxicity following the transition of T cells from a resting to an activated state, the effect of TCR restimulation on CD95L

upregulation and the triggering of AICD in previously activated T cells was investigated. The rationale for such an approach was that resting T cells are thought to be relatively insensitive to AICD (Wesselborg et al., 1993) since they have not yet upregulated CD95 expression (figure 4.11, A). Previously activated T cells in contrast express high levels of CD95 and thus can potentially receive signals via this route following the TCR-driven upregulation of CD95L expression (Wesselborg et al., 1993).

To investigate the process of AICD, SEB or anti-CD3 antibody was used for the provision of TCR signalling in previously activated T blasts. Since the induction of AICD has also been documented in Jurkat T cells, and the use of anti-CD3 antibody bypasses the need for TCR$\alpha\beta$ engagement, this approach was also taken in J16 cells. The key finding from this set of experiments is that both cell types were largely resistant to AICD. Consistent with the CD95 resistance documented in chapter 4, despite clear evidence that TCR engagement triggered the induction of functional CD95L expression in T blasts, there was nevertheless only a small (< 10%) increase in T cell apoptosis under these conditions. Thus, treatment with SEB or anti-CD3 allowed upregulation of CD95L which was presumably available for ligation of the CD95 receptor, and yet did not initiate an apoptotic pathway in the majority of T blasts as measured by JAM assay or TUNEL. It is interesting to note that even the most compelling data for a TCR-driven apoptotic response (SEB treatment of donor 1, figure 5.6) was characterised by apoptosis in only approximately 30% of the population, again arguing that the majority of these T cells were resistant to CD95-mediated death. In fact, the stimulation of cell cycle progression in T blasts following anti-CD3 treatment provides evidence that this event generated a predominantly positive signal under these circumstances, rather than promoting an apoptotic outcome despite the upregulation of CD95L expression.

The Jurkat T cells (J16) examined in this study also exhibited resistance to AICD in contrast with the findings of others (Takahashi et al., 1989; Dhein et al., 1995) possibly reflecting variation between Jurkat cell lines. In this regard, it is notable that Jurkat cell lines which do not exhibit anti-CD3 induced apoptosis have previously been reported (Zhu and Anasetti, 1995). Unlike the situation in T blasts, the lack of AICD in J16 cells appeared to be based on the failure to upregulate CD95L in response to TCR stimulation, rather than a lack of CD95 sensitivity in this cell line. The analysis of cell cycle changes highlighted the differential response to anti-CD3 treatment exhibited by J16 cells compared to T blasts: whilst J16 cells underwent cell cycle arrest under these conditions, accumulating in G0/G1 region of the DNA histogram, T blasts in contrast exhibited increased S-phase entry following TCR signalling. This finding is in line with other studies which have documented anti-CD3 induced growth arrest rather than apoptosis induction in Jurkat cell lines (Zhu and Anasetti, 1995).

Data from murine studies have indicated that, in contrast with these observations in T blasts, anti-CD3 treatment resulted in apoptosis of the majority of activated T cells (74% apoptosis) (Ettinger et al., 1995) and similarly murine T cell hybridomas were largely sensitive to apoptosis via this route (Brunner et al., 1995; Ju et al., 1995; Yang et al., 1995). Published support for the process of AICD in human T cells is more limited and whilst approximately 50% sensitivity to anti-CD3 induced apoptosis has been documented (Groux et al., 1993), work by Krammer and colleagues (Dhein et al., 1995) demonstrated apoptosis in only a minority of T cells (less than 30% in most experiments) following anti-CD3 treatment, despite the authors' interpretation of these data as evidence for apoptosis sensitivity. AICD has also been documented in human T cell clones (Alderson et al., 1995; Hargreaves et al., 1997) although again there is variation in the degree of cell death monitored with the apoptotic response being confined to approximately 45% in the former study. Moreover, since it has been demonstrated here that T cell clones do not necessarily reflect the responses of

peripheral blood T cells with regard to CD95 sensitivity (figures 4.20, 4.21) it is likely that the propensity to undergo AICD is also altered in these cells. Similarly, results from murine T cell hybridomas cannot necessarily be extrapolated to normal human T cells since the cell death may not be identically regulated in these cells. For example the substantial CD95L surface staining of TCR-stimulated A1.1 murine cells using the CD95-Fc protein is notable in the report by Brunner and colleagues (Brunner et al., 1995), but equally unexpected in this study is the extremely low CD95 receptor expression even following cellular activation, possibly indicating that control of receptor expression may be more important than regulation of ligand availability for the control of the CD95 pathway in this particular T cell hybridoma.

In vivo work on AICD has utilised SEB injection allowing the responding cells to be tracked by virtue of their distinct TCR-Vß chains. In this system, SEB-reactive T cells first proliferate then are deleted by apoptosis, such that they have been removed from the mouse by 2 weeks following immunisation (MacDonald et al., 1991). The mechanism of T cell elimination is not defined in these studies and whilst *in vitro* work using CD4+ T cells from MRL +/+, MRL-*lpr* and MRL-*gld* mice has indicated that SEB-induced apoptosis is CD95-dependent (Ettinger et al., 1995), the availability of costimulatory ligands in the SEB activation step is not clear in these experiments and may not precisely reflect *in vivo* events. In addition, it is difficult to address whether the *in vivo* approach of high dose (10mg) SEB injection accurately models the immune response to invading pathogens, since there may be key differences in the location and nature of antigen presentation events, the induction of costimulatory ligands and the persistence of antigen. T cell recognition of peptide antigen is known to involve APC-mediated processing such that discontinuous epitopes are presented in the context of the HLA antigen-binding groove. In contrast, superantigens bypass conventional recognition requirements by interacting outside of the specificity-determining regions on both TCR and HLA (Herman et al., 1991; Dohlsten et al., 1993;

Kappler et al., 1989), and can even bind directly to the T cell antigen receptor in the absence of HLA (Lando et al., 1993). Thus, the mechanisms which normally restrict which T cells respond to a particular challenge are clearly not applicable to superantigen-driven activation, and the nature of the cells acting as APCs in such *in vivo* responses has not been defined. Moreover, the kinetics of antigen clearance may differ in this system since it is unclear how long superantigens remain associated with the TCR and it is possible that persistent signalling via this route may contribute to apoptosis induction. In support, it has been observed that T cell elimination may be preferentially associated with superantigen- rather than peptide- induced responses (Weber et al., 1995). Thus *in vivo* SEB studies offer a powerful tool for examining T cell activation in the context of a multi-cell type immune response, however, whilst the observed SEB-induced deletion is likely to be CD95-mediated (AICD), this remains to be formally demonstrated.

The fact that human T cells appeared less sensitive to AICD via CD95 than their murine counterparts raises the possibility that the CD95 system may be differentially regulated in these two species. In support of this concept, there are a number of species-specific differences pertaining to the molecules which mediate this process. Accordingly it has been demonstrated that the murine form of soluble CD95L is largely inactive whilst the human form of this protein exhibits potent cytotoxicity (Tanaka et al., 1995) rendering it potentially dangerous to cells which are constitutively sensitive to CD95 ligation. The absence of functional soluble CD95L in the murine system may explain why naive T cells express higher levels of surface CD95 in mice compared to humans (Ettinger et al., 1995) and are constitutively sensitive to apoptosis via CD95L (Suda et al., 1996). A more fundamental difference may relate to the activation-induced regulation of CD95 expression in the two systems: whilst human T cells exhibit stable CD95 expression following activation (Owen-Schaub et al., 1992), in murine T cells surface expression of this receptor is subject to greater modulation with initial activation-dependent upregulation being followed by subsequent downregulation

(Ettinger et al., 1995; Tucek-Szabo et al., 1996). It is thus conceivable that the CD95 system may be preferentially regulated by control of CD95 receptor expression in mice whilst the control of sensitivity to CD95 ligation might achieve the same aim in humans.

In summary, whilst the induction of apoptosis was clearly not a major response following TCR ligation in T blasts, the data provided evidence that the first part of the AICD process, namely the induction of CD95L expression, was intact in these cells. This supported the notion that the stimuli used to mimic antigen engagement of the TCR (SEB or anti-CD3) were competent to deliver the requisite signals for this process, and further evidence for reagent efficacy was provided by the calcium mobilisation data (figure 5.9) in addition to the effective induction of proliferation (figure 3.2). The lack of apoptosis in these cultures, therefore, could not be attributed to a lack of CD95L upregulation, but rather appeared to reflect the insensitivity of activated T cells to the induction of death via the CD95 pathway.

CHAPTER 6

Protection from CD95-Mediated Apoptosis

6.1 INTRODUCTION

The experiments presented in Chapters 4 and 5 indicated that protective mechanisms were clearly operating in normal activated T cells to prevent the induction of apoptosis following CD95 ligation. This view is not widely accepted, even though resistance to apoptosis is evident in part of the population (often the majority) in nearly all the apoptosis studies published (Miyawaki et al., 1992; Klas et al., 1993; Alderson et al., 1995; Brunner et al., 1995; Dhein et al., 1995; Peter et al., 1997). In choosing to ignore those cells which are resistant to CD95-mediated killing, it is possible that the most interesting biological question is being overlooked in such studies, namely the control of the CD95 pathway under normal circumstances. Understanding the regulation of the sensitivity of T cells to apoptosis is of potential therapeutic relevance since the ability to manipulate this phenotype would confer enormous clinical potential for example in the context of autoimmunity where the elimination of a subset of autoreactive immune cells may be desirable. Therefore, this chapter presents experiments carried out to investigate the nature of the CD95 resistance evident in T blast cultures and examines the circumstances under which this resistance might be conferred under physiological conditions.

To probe the protective potential of various experimental treatments, the constitutively CD95 sensitive Jurkat T cells were utilised as a model for investigating the control of CD95 resistance. Accordingly, Jurkats were subjected to experimental treatments with a view to inhibiting the process of CD95-mediated apoptosis and providing information about how this process might be controlled to generate CD95 resistance in T blasts.

6.2 RESULTS

6.2.1 Role of Antigen-like Signals

It has been established that during T cell activation, the surface expression of both CD95 and CD95L is upregulated, therefore it is likely that antigen-derived signals via the TCR may accompany CD95 engagement under these circumstances. To investigate whether the provision of TCR-like signals could modulate the response to CD95 ligation in Jurkat T cells, the phorbol ester PMA was used as an activator of PKC. J16 cells were subjected to a 1 hour pre-incubation with PMA prior to a 4 hour treatment with the apoptotic anti-CD95 antibody CH11, following which apoptosis was assessed by annexin-FITC binding (figure 6.1). This analysis revealed that the extent of apoptosis induced by CH11 treatment was markedly reduced in the presence of PMA, since the percent annexin-stained cells decreased from 55.8% to 39.7%. Inclusion of PMA therefore lead to a 32.6% inhibition of CH11-induced apoptosis in this experiment, as measured by PS exposure and analysis of results from 5 independent experiments indicated that the inhibition of CD95-mediated apoptosis by PMA was statistically significant ($p < 0.05$).

In order to further investigate the ability of PMA treatment to inhibit CD95-mediated apoptosis, the effect of titrating the concentration of PMA on apoptosis induction through the CD95 pathway was examined in the JAM assay (figure 6.2). In addition, the effect of the non-PKC -activating phorbol ester 4α-phorbol was also investigated as a control for PMA treatment. ^{3}H-thymidine labelled J16 cells were therefore pre-incubated with the indicated concentration of PMA or 4α-phorbol for 1 hour prior to a 4 hour treatment with CH11. These data revealed that the inhibition of apoptosis by PMA was dose dependent and reproducible, although this treatment did not necessarily prevent apoptosis in the whole population. In contrast 4α-phorbol did not detectably inhibit CD95-mediated apoptosis at any of the concentrations utilised, indicating that PKC activity was a

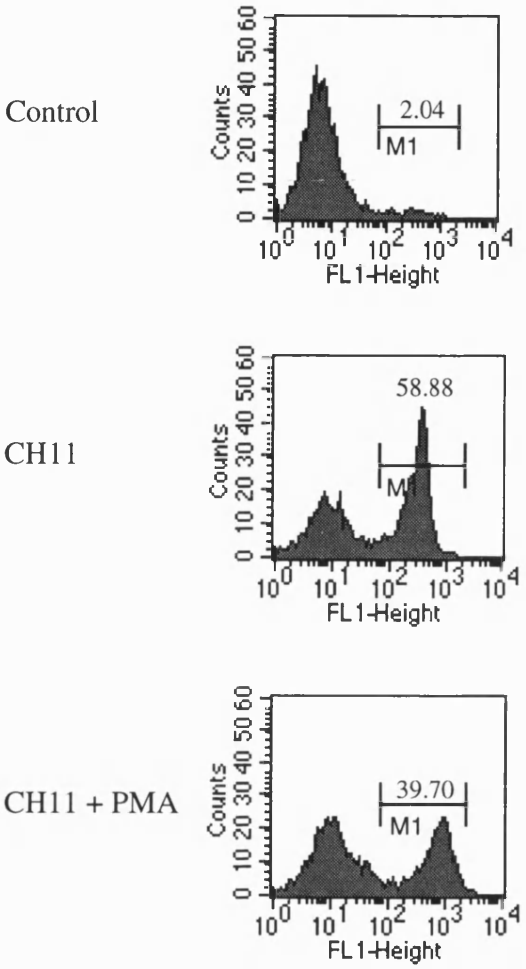


Figure 6.1: Effect of PMA on CH11-induced apoptosis of T cells. J16 cells were pre-incubated for 1h with PMA (0.04µg/ml) then treated for 4h with the anti-CD95 antibody CH11 (0.1µg/ml). Apoptosis was measured by annexin-FITC binding and is visualised as an increase in fluorescence in the gated region. Presented histograms are representative of 5 separate experiments and pooled data indicate that inhibition of CH11-mediated apoptosis by PMA was significant at the level of $p < 0.05$. Mean values (+/- SEM) for pooled data were as follows: control = 4.3 (+/- 2.1), CH11 = 50.6 (+/- 8.7), CH11 + PMA = 32.0 (+/- 6.2).

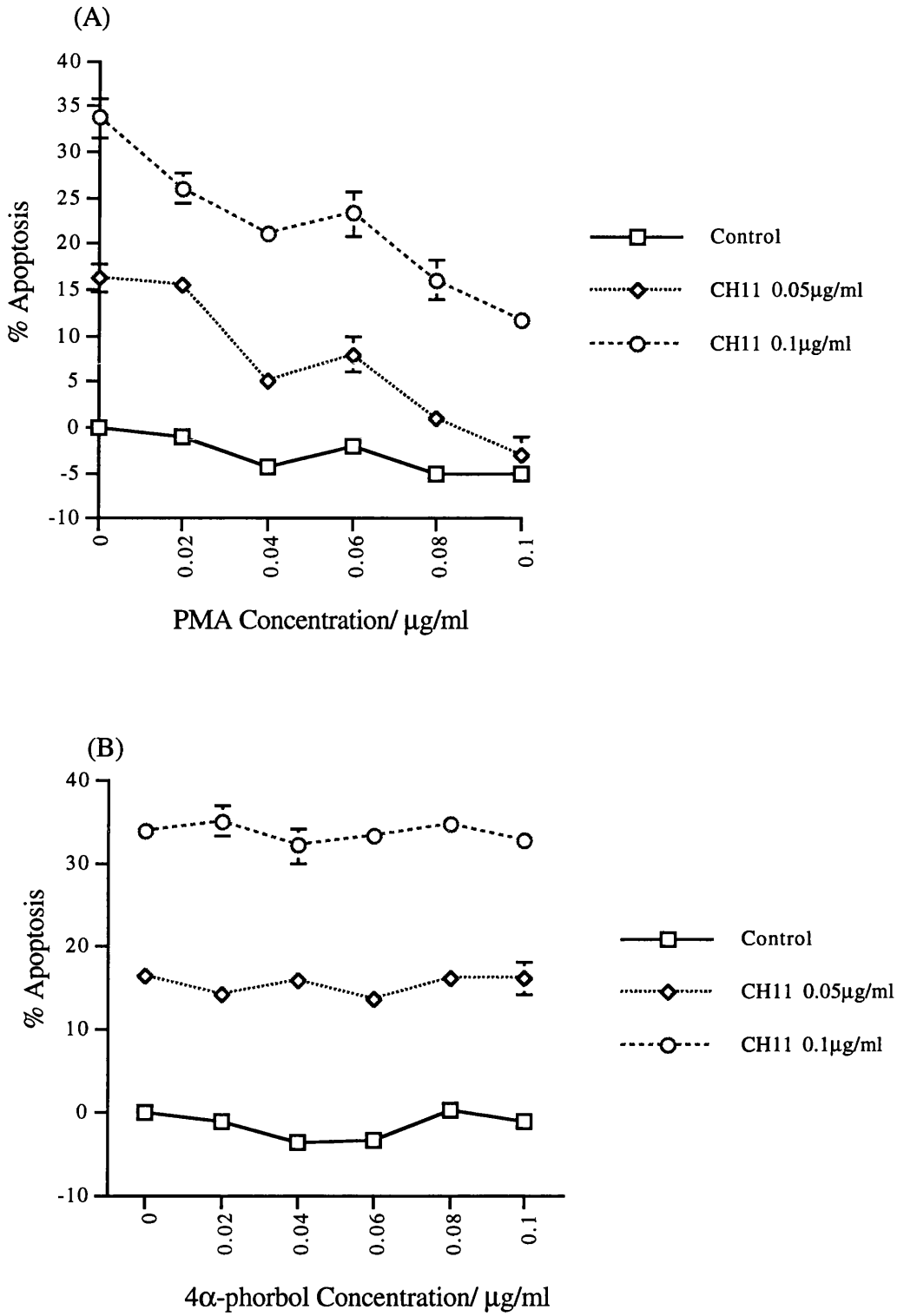


Figure 6.2: Effect of PMA and 4α-phorbol on CD95-induced apoptosis. ^{3}H-thymidine-labelled J16 cells were pre-treated for 1h with PMA (A) or 4α-phorbol (B) and exposed to the anti-CD95 antibody CH11 for 4h. Apoptosis was measured by JAM assay and the mean (+/-SEM) of triplicate wells is shown. Apoptosis was calculated as the % decrease in CPM relative to control-treated cells. Data are representative of 3 experiments.

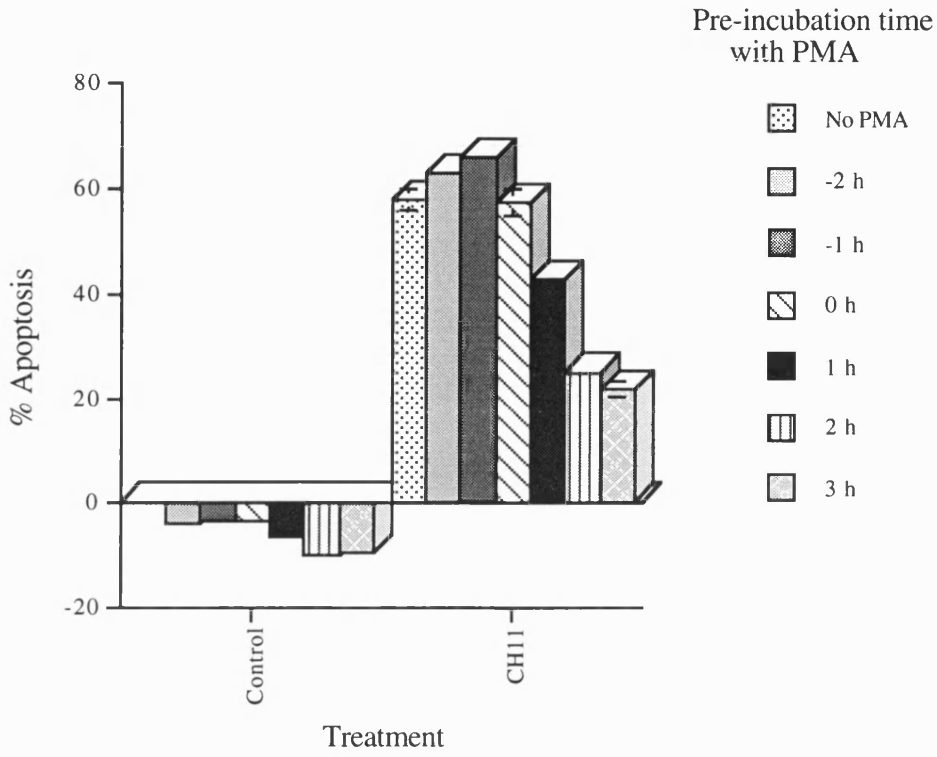


Figure 6.3: Effect of varying the timing of PMA addition on CD95-induced apoptosis. ^{3}H-thymidine labelled J16 cells were pre-incubated for the indicated time period relative to the addition of the anti-CD95 antibody CH11 (thus -2h indicates PMA addition 2h after the commencement of CH11 treatment). Following a 4h incubation with CH11 (0.1µg/ml) cells were harvested and apoptosis was assessed by JAM assay. The mean (+/-SEM) of triplicate wells is shown and % apoptosis is calculated relative to control-treated cells. Data are representative of 3 experiments.

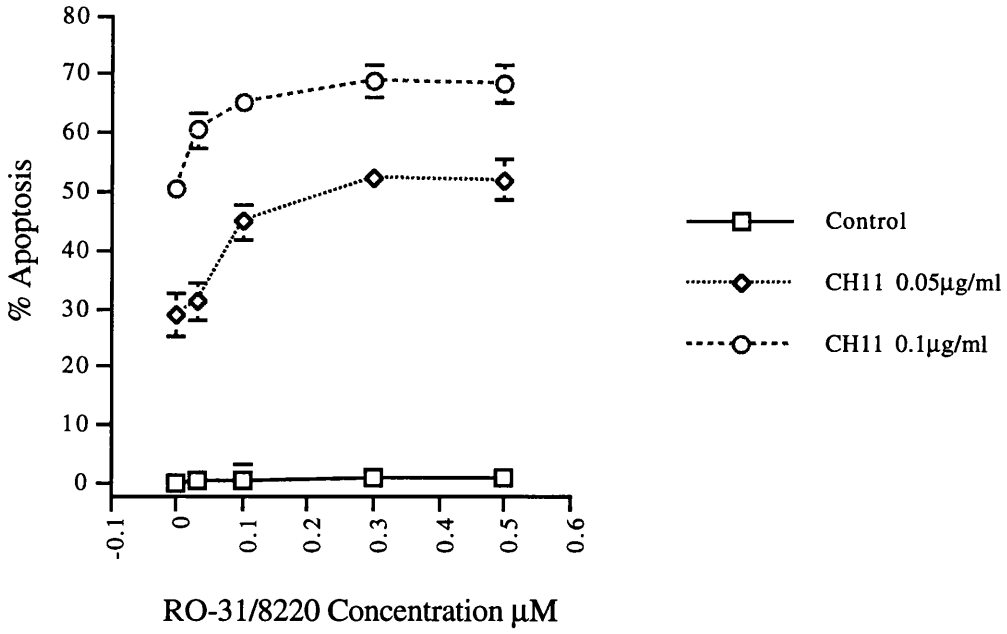


Figure 6.4: Effect of the PKC inhibitor RO-31/8220 on CD95-mediated apoptosis. ^{3}H-thymidine labelled J16 cells were pre-incubated for 1h with the PKC inhibitor RO-31/8220 then exposed to the anti-CD95 antibody CH11 for 4h. Apoptosis was measured by JAM assay and was calculated relative to control-treated cells. The mean (+/-SEM) of triplicate wells is shown and presented data are representative of 5 independent experiments from which pooled data indicate that the potentiation of CH11-induced apoptosis (0.05µg/ml) by RO-31/8220 (0.5µM) was significant at the level $p < 0.05$. Mean values (+/- SEM) for pooled data were as follows: control = 2.7 (+/- 1.5), CH11 = 30.0 (+/- 5.1), CH11 + RO-31/8220 = 47.3 (+/- 5.5).

requirement for the observed protection. Toxicity controls using PI exclusion assay indicated that the viability of J16 cells was not compromised by PMA or 4α-phorbol treatment, even at the highest dose (0.1μg/ml) during these 5 hour assays. Kinetic analyses indicated a requirement for pre-incubation with PMA to allow detectable protection from CD95-mediated apoptosis, with the effect plateauing at the 2 hour pre-incubation time point (figure 6.3). Addition of PMA at the same time as (or after) CH11 treatment did not observably reduce the extent of apoptosis.

Since PKC activation appeared to inhibit CD95-mediated apoptosis, it was also of interest to investigate the effect of the PKC inhibitor RO-31/8220 (Davis et al., 1989) on this pathway (figure 6.4). At the concentrations of RO-31/8220 utilised, this compound alone did not induce apoptosis, but when used in combination with CH11 resulted in marked potentiation of the apoptotic response. For example the percentage apoptosis when 0.1μg/ml CH11 was used increased from 28.9% to 52% if cells were first pre-incubated with 0.5μM RO31/8220 (figure 6.4). Analysis of data from 5 independent experiments revealed that the potentiation of CD95-mediated apoptosis by RO31/8220 was statistically significant ($p < 0.05$) when 0.05μg/ml CH11 and 0.5μM RO31/8220 were used.

Since PMA appeared to protect J16 cells from CD95-mediated apoptosis, the effect of this treatment on apoptosis in SEB blasts was also examined in order to assess whether this mechanism of protection may be of relevance to normal T cells. The experiment presented in figure 6.5 illustrates one of the larger apoptotic responses observed in T blasts (32%) although, consistent with earlier results, this response was still limited to a minority of the population. Inclusion of PMA with the CH11 treatment, however, markedly reduced the degree of apoptosis induction as measured by annexin-FITC binding, implying that receptors which activate PKC might play a role in protection from apoptosis in normal T cells.

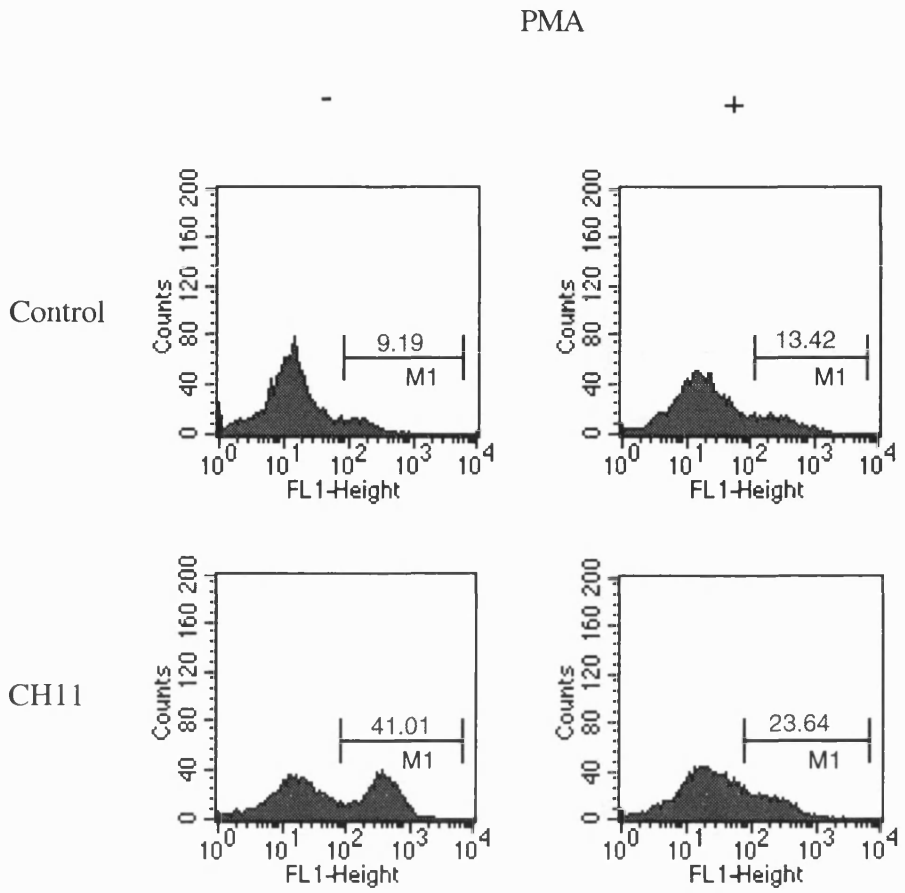


Figure 6.5: Effect of PMA on CH11-induced apoptosis in T blasts. Day 6 SEB blasts were incubated for 15h with the anti-CD95 antibody CH11 (0.5µg/ml) in the presence of PMA (0.04µg/ml) where indicated. Apoptosis was measured by annexin-FITC binding. A CD95-dependent increase in fluorescence of 31.82% was observed in the absence of PMA compared to 10.22% in the presence of PMA. Data are representative of 3 experiments.

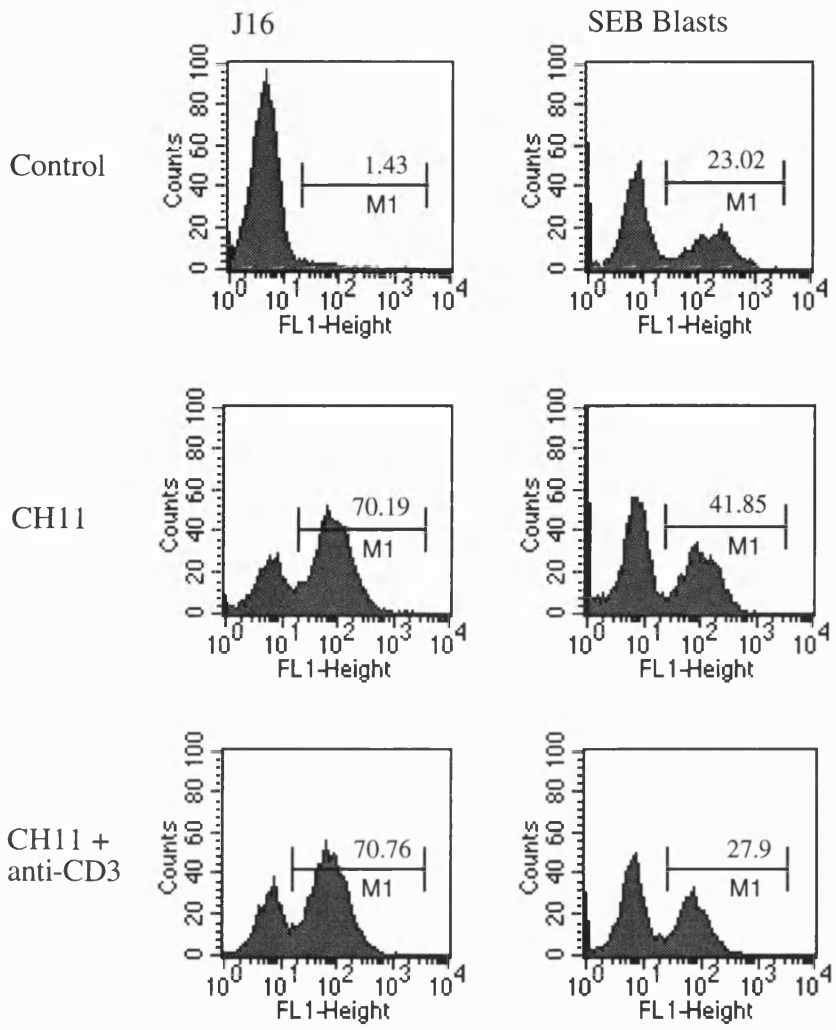


Figure 6.6: Effect of anti-CD3 treatment on CH11-induced apoptosis in T cells. J16 cells or day 6 SEB blasts were treated for 15h with the anti-CD95 antibody CH11 (0.5µg/ml) in the presence of immobilised anti-CD3 (plate-coated at 10µg/ml) where indicated. Apoptosis was measured by annexin-FITC binding. CH11-induced increases in fluorescence of 68.76% (in the absence of anti-CD3) and 69.33% (in the presence of anti-CD3) were observed for J16 cells and 18.83% (in the absence of anti-CD3) and 4.88% (in the presence of anti-CD3) were measured for SEB blasts. Data are representative of 3 independent experiments.

Given that a major signal which drives PKC activation in T cells is ligation of the TCR (Genot et al., 1995), the effect of anti-CD3 signalling on CD95-induced apoptosis was also assessed. Accordingly both J16 cells and SEB blasts were exposed to immobilised anti-CD3 prior to exposure to the anti-CD95 antibody CH11. Interestingly, whilst in J16 cells this did not result in protection, SEB blasts in contrast exhibited a marked reduction in the apoptotic response (virtually to baseline levels) in the presence of anti-CD3 with the percent annexin-stained cells decreasing from 41.85 to 27.9% under these conditions (background apoptosis was 23.02%) (figure 6.6). These data suggested that signalling through the TCR offered one potential mechanism for the prevention of CD95-mediated apoptosis in T cells. Such a mechanism would explain how activated T cells could survive despite upregulated expression of both CD95 and its ligand.

6.2.2 Role of Costimulatory Signals

As indicated previously (chapter 3), during the course of T cell activation CD28 is both downregulated by CD80 as well as subsequently upregulated in a manner which is dependent on the provision of TCR-like signals. Early activated T cell cultures are therefore heterogeneous for CD28 expression whilst expression of CD95 is nevertheless homogeneous (this point has been previously illustrated in figure 3.4, panel B). This heterogeneity therefore provides a useful system in which to assess the significance of CD28 expression on T cell responses. Accordingly, early activated T cell populations were treated with the apoptotic anti-CD95 antibody CH11 and dual stained for TUNEL positivity and CD28 expression with a view to discerning differential responses between the CD28 high and CD28 low populations (figure 6.7). Interestingly, this analysis revealed that cells expressing high levels of CD28 were resistant to CD95-mediated apoptosis since there was no decrease in the CD28 high annexin dull (upper left) population following CH11 treatment. In contrast, the cells which have

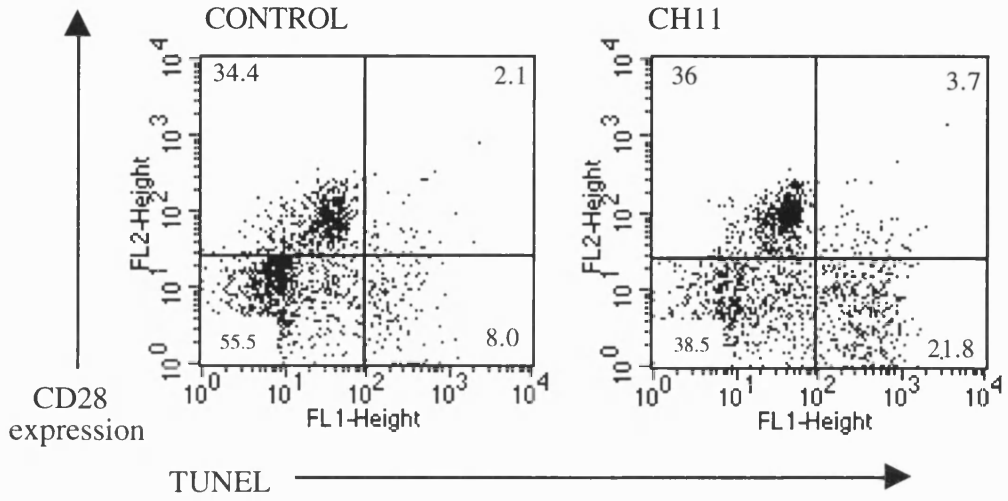


Figure 6.7: Simultaneous analysis of CD28 expression and apoptosis following CH11 treatment of T blasts. Day 3 SEB blasts were incubated for 15h with the anti-CD95 antibody CH11 (0.5µg/ml) then stained for CD28 expression (FL2-fluorescence) and assayed for apoptosis induction by TUNEL analysis (FL1-fluoresence). Data are representative of 3 similar experiments.

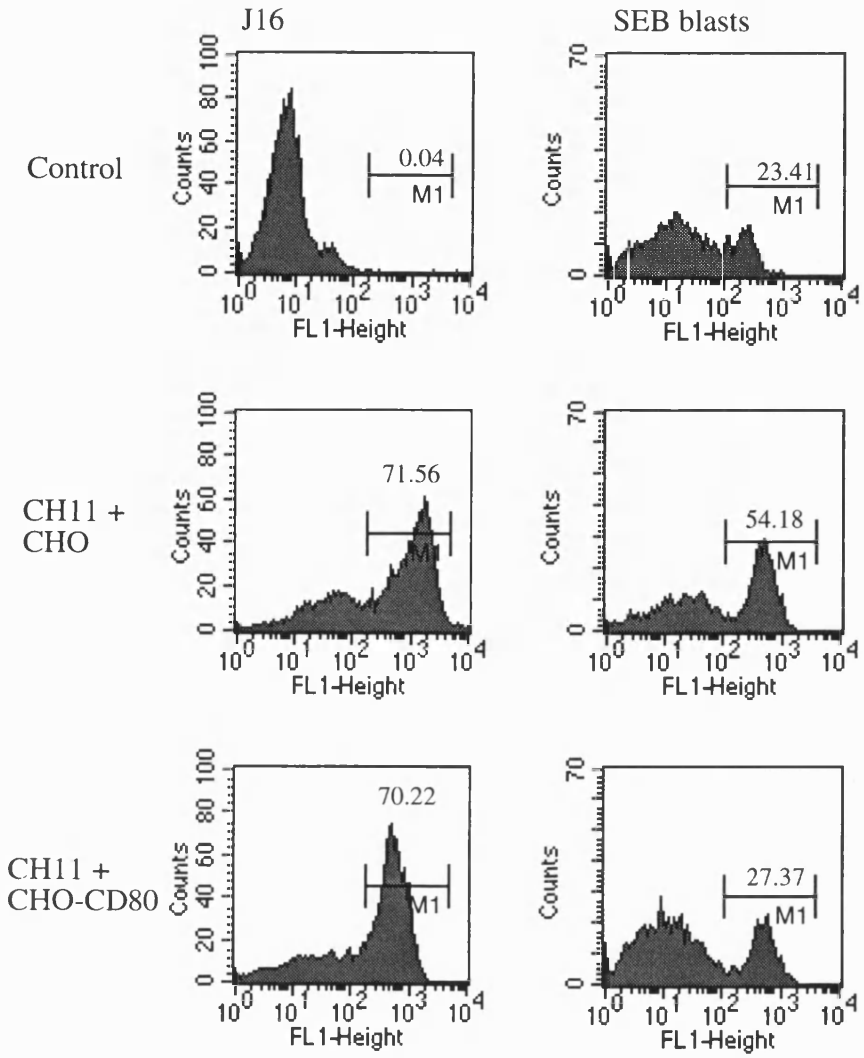


Figure 6.8: Effect of CD80 transfectants on CH11-induced apoptosis in T cells. J16 cells or day 6 SEB blasts were incubated for 15h with the anti-CD95 antibody (CH11) in the presence of CD80 transfectants or untransfected CHO cells. Apoptosis was measured by annexin-FITC binding. J16 cells exhibited a CH11-induced increase in fluorescence of 71.52% (in the presence of CHO cells) and 70.18% (in the presence of CD80 cells). SEB blasts exhibited a CH11-induced increase in fluorescence of 30.77% (+ CHO cells) and 3.96% (+ CD80 cells). Data are representative of 4 similar experiments.

undergone apoptosis in this experiment can be seen to have derived from the CD28 low population of starting cells. These data indicated that low CD28 expression pre-disposed to CD95 sensitivity in these cultures and that conversely, T cells which expressed high levels of CD28 were protected from CD95-mediated apoptosis. Moreover, the demonstration that CD28 high cells were protected from apoptosis gave the first indication that expression of high levels of CD28 might potentially offer a mechanism for the delivery of anti-apoptotic signals.

To test this hypothesis directly, J16 cells and SEB blasts were exposed to CH11 in the presence or absence of CHO cells transfected with CD80 (figure 6.8). These results demonstrated that whilst J16 cells could not be protected via this route (under these experimental conditions), SEB blasts underwent substantially less apoptosis if exposed to the ligand for CD28 simultaneously with the anti-CD95 antibody CH11. Accordingly the percentage of annexin-stained cells decreased from 54.18% to 27.37% (background apoptosis was 23.41%) upon inclusion of CD80 transfectants in this experiment. This indicated that signalling via the CD28 receptor stimulated anti-apoptotic activities. Whilst a role for CD28 in T cell survival has been inferred from a number of studies (Boise et al., 1995; Noel et al., 1996a; Sperling et al., 1996; Vella et al., 1997), direct evidence of this nature has been lacking and this appears to be the first demonstration of a direct role for CD28 ligation in the prevention of CD95-mediated apoptosis in normal human T cells.

Since CD28 ligation could protect from apoptosis, this prompted an analysis of the expression of the CD28 ligands CD80 and CD86 in T blasts cultures, since inducible T cell expression of these molecules has been documented (Sansom and Hall, 1993; Pichler and Wyss-Coray, 1994). Resting human T cells failed to express detectable levels of CD80 and CD86, and the first stimulation with SEB only triggered marginal CD86 upregulation (figure 6.9). Subsequent restimulations, however, resulted in a sequential increase in the expression of

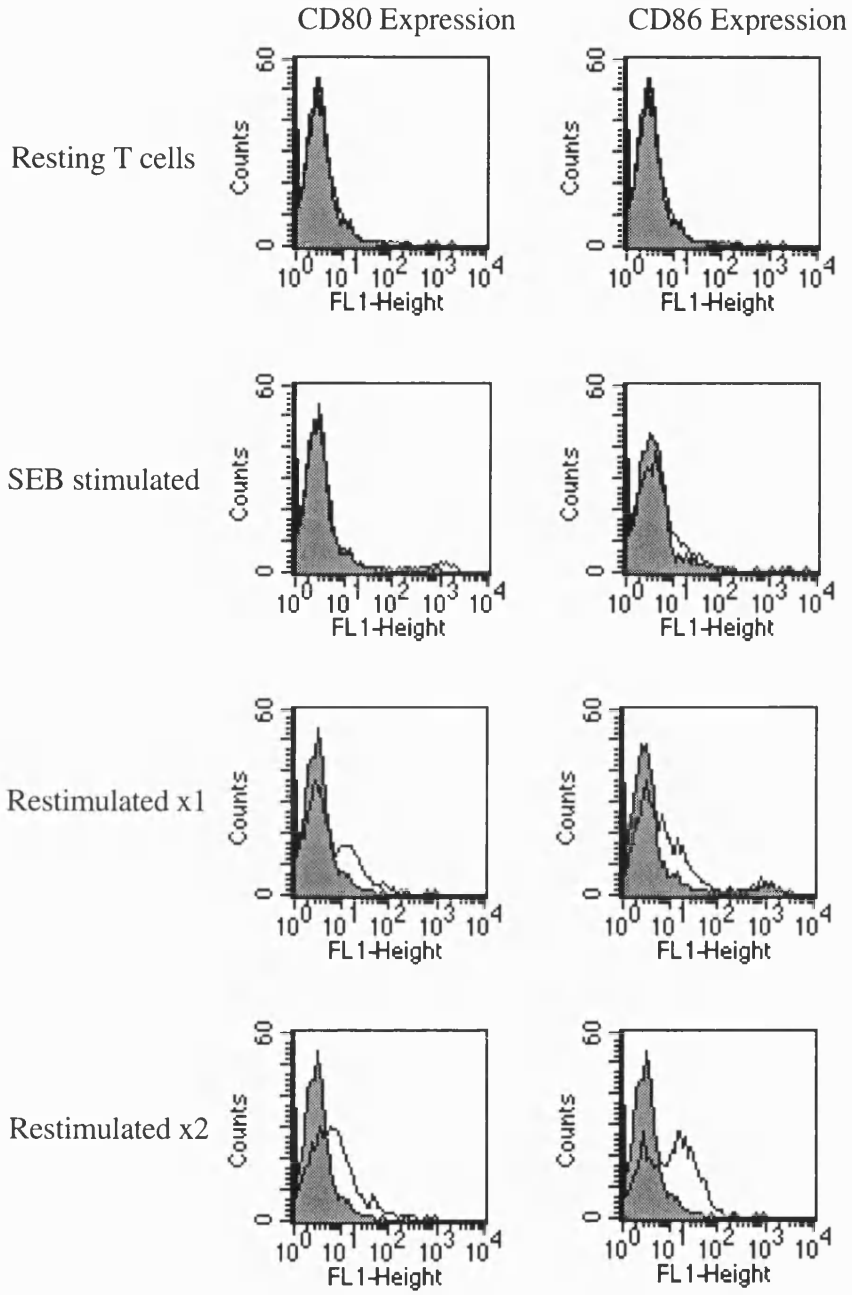


Figure 6.9: Expression of CD80 and CD86 on T cells. Resting human T cells, or SEB blasts restimulated with SEB as indicated, were stained for surface expression of CD80 and CD86 (open histograms). Filled histograms indicate control staining with secondary antibody only. Data are representative of 2 similar experiments.

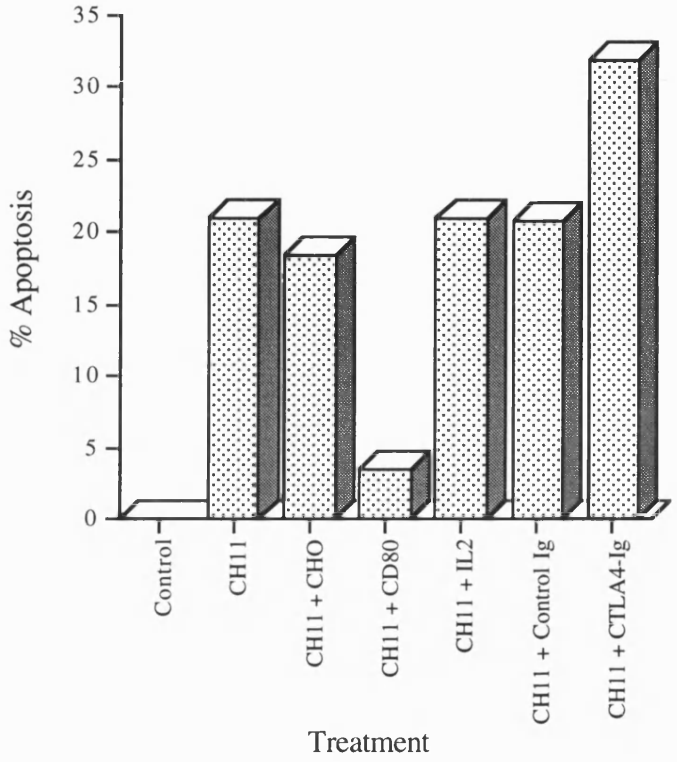


Figure 6.10: Reciprocal effect of CD80 transfectants and CTLA4-Ig treatment on CH11-induced apoptosis in T blasts. Day 6 SEB blasts were incubated for 15h with the anti-CD95 antibody CH11 (0.5μg/ml) in the presence of CD80 transfectants, untransfected CHO cells, control Ig (human Ig, 2μg/ml), CTLA4-Ig (2μg/ml) or IL-2 (10 IU/ml) as indicated. Apoptosis was measured by annexin-FITC binding and the % increase in fluorescence relative to control-treated cells is shown. This experiment was repeated 4 times and similar trends were observed.

these molecules as illustrated in figure 6.9. In particular, CD86 was expressed at substantial levels in repeatedly activated T cells. Thus one interaction which might confer a CD95-resistant phenotype on activated T cells is the ligation of CD28 by CD80/86 expressed on T cells. To investigate the contribution of such an interaction to the CD95 resistance observed in T blasts, signalling via the CD28 receptor (by T cell CD80/86) was blocked by the inclusion of CTLA4-Ig during the incubation with CH11 and the effect on apoptosis induction was assessed by annexin-FITC binding (figure 6.10). This analysis revealed that inclusion of CTLA4-Ig during CH11 incubations resulted in potentiation of CD95-mediated apoptosis, indicative of a protective role for T cell CD80 family members. The extent of CTLA-Ig-induced potentiation of CD95-mediated apoptosis was variable between cultures with up to 30% potentiation being observed under these experimental conditions. It is likely that pre-incubating cultures with CTLA4-Ig prior to the anti-CD95 challenge might result in more substantial potentiation of apoptosis and this therefore represents a target for further investigation. The variability in the degree of potentiation observed may reflect the variable expression levels of CD80/86 on different T blast lines. Overall these data suggested that CD28 ligation offered one route for the protection of T cells from CD95-mediated apoptosis, and that T cell expression of CD80/86 could potentially play a role in regulating this process.

The nature of the anti-apoptotic signal transduced by CD28 is not yet clear. Since a feature of CD28 signalling is enhanced cytokine production (Gimmi et al., 1991; Jenkins et al., 1991; Linsley et al., 1991a; Razi-Wolf et al., 1992; Sansom et al., 1993), one possibility is that protection could be mediated by IL-2 production, therefore the effect of the addition of exogenous IL-2 on apoptosis induction of was assessed. As indicated in figure 6.10, IL-2 failed to inhibit CD95-mediated apoptosis implying that the protection conferred by CD28 engagement could not be attributed to the consequent enhanced production of this cytokine. This finding

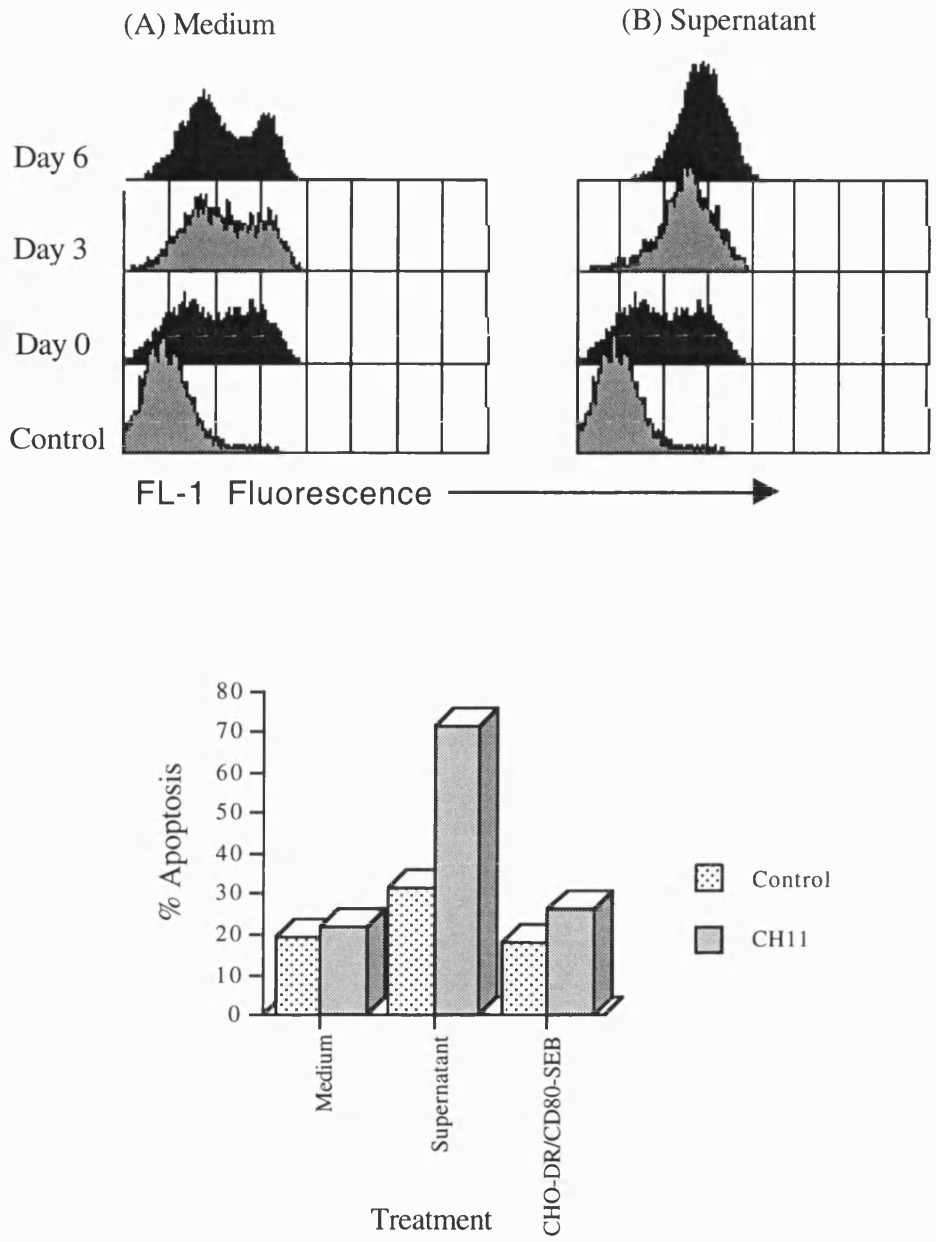


Figure 6.11: CD95 expression and apoptosis sensitivity in T cells activated by supernatant transfer. Supernatants from day 3 T blasts (activated using anti-CD3 and CD80 transfectants) were transferred to resting purified T cells (day 0). CD95 expression of supernatant-treated T cells was measured on days 3 and 6 and compared with that of cells incubated in medium (upper panel). Sensitivity to CD95-mediated apoptosis (0.05μg/ml CH11, 15h) was assessed on day 6 by annexin-FITC binding and compared with cells treated with medium or SEB (pulsed onto CHO-DR/CD80) (lower panel). Apoptosis is shown as the % annexin bright cells in each sample. Data are representative of 3 independent experiments.

is in line with reports indicating a lack of effect of IL-2 on CD95-induced apoptosis (Zipp et al., 1997).

Since signalling via the TCR CD3 complex (figure 6.6) and via the CD28 receptor (figures 6.8 and 6.10) appeared to provide protection from CD95-mediated apoptosis in T blasts, this suggested that "correct" activation signals (i.e. antigen plus costimulation) might play a role in promoting T cell survival. In order to address the role of TCR and CD28 signalling in the protection of T cells from CD95-mediated apoptosis, experiments were undertaken in which the provision of these signals were deliberately bypassed. Accordingly, resting purified T cells were "activated" by culture in the supernatants from T blasts which had been stimulated for 3 days with immobilised anti-CD3 in the presence of CD80 transfectants. Surface staining of T cells treated with supernatants from activated T cells revealed upregulated expression of the CD95 receptor (figure 6.11, upper panel) therefore sensitivity to CD95 ligation was examined and compared both with unstimulated T cells and with HLA-DR/SEB/CD80 activated cells. This analysis revealed that despite equivalent CD95 expression, T cells activated by supernatant transfer showed increased sensitivity to CD95-mediated apoptosis compared to T cells stimulated with HLA-DR/SEB/CD80. These data support the contention that TCR and CD28 signalling offer protection from CD95-induced apoptosis and suggest that ineffective activation predisposes to apoptosis.

6.3 DISCUSSION

Based on the observation that normal activated T cells were largely resistant to the induction of apoptosis via the CD95 pathway, an investigation of the nature of the stimuli which may be involved in maintaining this resistance was initiated. The data presented indicate that one contributory factor to the control of CD95-

mediated apoptosis was the activation status of cellular PKC, such that stimulation with the phorbol ester PMA inhibited the apoptotic response, whilst the PKC inhibitor RO-31/8220 in contrast potentiated this pathway. These findings are supported by previous studies which have demonstrated a role for PKC-activating phorbol esters in the promotion of T cell survival (Tomei et al., 1988; Rodriguez-Tarduchy and Lopez-Rivas, 1989; Lucas et al., 1994). A key pathway for the triggering of PKC activation in T cells is ligation of the TCR (Genot et al., 1995) implying that this may represent one route by which sensitivity to CD95-mediated apoptosis may be modulated. Interestingly, whilst this hypothesis was not supported by the studies in Jurkat cells, it did prove to be the case in superantigen activated peripheral blood T cells, with anti-CD3 treatment resulting in reproducible inhibition of CH11-induced cell death. One factor highlighted by these studies, therefore, was that although certain biological responses are conserved between Jurkats and normal T cells (e.g. protection from apoptosis by PMA), these homologies are not universally applicable and the limitations of Jurkat cell lines as a model of normal human T cells should be understood.

For SEB blasts, therefore, a signal through the CD3 component of the T cell receptor was sufficient to markedly inhibit the apoptotic response to CD95 engagement. These data contrast with the reports documenting AICD following anti-CD3 treatment of human T cells (Groux et al., 1993; Alderson et al., 1995; Dhein et al., 1995), and suggest that under these conditions TCR signalling was promoting T cell survival rather than triggering an apoptotic programme. Since the percentage of cells induced to undergo apoptosis in the above reports was relatively low (less than 50%), one possibility is that CD3 signalling can trigger both apoptosis and survival depending on the context in which signalling is delivered. T cell populations clearly exhibit a degree of heterogeneity and the choice between life and death within an individual T cell following TCR signalling may be influenced by factors such as the activation or differentiation state. Thus the observed outcome of anti-CD3 treatment at the population level

may represent the net result of both apoptosis induction and survival signalling. Given that the data presented in this chapter indicate a role for "correct" activation signals in the promotion of cell survival, the variation in the extent of AICD observed between reports (Groux et al., 1993; Alderson et al., 1995; Dhein et al., 1995; Boshell et al., 1996) could potentially reflect the nature or effectiveness of the initial activation stimulus.

The inhibition of apoptosis by TCR signalling is in line with recent studies on B lymphocytes which demonstrated the promotion of cell survival following antigen-receptor engagement (Rothstein et al., 1995; Foote et al., 1996) and is further supported by the recent association of TCR signalling with T cell survival in murine systems (Suda et al., 1996; Kirberg et al., 1997). The duration of TCR-mediated protective signals following T cell antigen engagement may thus define a window of time for T cell immune functions to proceed prior to the acquisition of sensitivity to apoptosis. The persistence of antigen, for example due to incomplete elimination of pathogens or immune complex retention on germinal centre follicular dendritic cells, is believed to contribute to immune memory (Ahmed and Gray, 1996; Sprent et al., 1997) and is in line with the finding that anti-CD3 treatment can promote T cell survival.

Consistent with a role for activation signals in protection from T cell death, further experiments indicated that signals delivered by CD80 could also protect SEB blasts from CD95-mediated apoptosis. Again, parallel studies in Jurkat cells failed to mimic this phenomenon, highlighting the possible dysregulation of certain signalling pathways in transformed cell lines. The degree of protection conferred by CD28 ligation in T blasts varied between donors (as did the degree of apoptosis in response to CD95 ligation, and the degree of potentiation afforded by CTLA4-Ig treatment) indicating that additional factors such as the CD80/86 expression and activation/differentiation status of these cells may influence such responses.

CD80-mediated protection from apoptosis was not mimicked by exogenous addition of IL-2, indicating that the anti-apoptotic signal(s) induced was not mediated by this cytokine. Protection from AICD by CD80 is not likely to occur by inhibiting the induction of CD95L since a) experiments described in the previous chapter illustrated equivalent CD95L induction regardless of CD80 provision (figure 5.10) and b) protection was demonstrated to be downstream of CD95 engagement in experiments using CH11 for direct CD95 ligation (figures 6.8 and 6.10). Upregulation of $BCLX_L$ represents one candidate for mediating the CD80-induced protective effect, although transfection studies have indicated that this protein can inhibit CD95-mediated apoptosis in Jurkats (Boise et al., 1995) but not normal T blasts (Moreno et al., 1996). Previous work in our laboratory has established that ligation of CD28 alone in activated T cells is sufficient to trigger induction of the transcription factor Nuclear Factor κB (NFκB) (Edmead et al., 1996) which has recently been implicated in protection from TNFR-induced apoptosis (Liu et al., 1996; Baichwal and Baeuerle, 1997). Given the homologies between CD95- and TNFR-induced signalling (Boldin et al., 1996; Cleveland and Ihle, 1995), it is possible that NFκB could represent one potential target for CD28-mediated survival effects. Alternatively, CD28 stimulation is known to lead to the recruitment and activation of the lipid kinase PI3K (Ward et al., 1993; Cai et al., 1995) which has been linked to the activation of the serine/threonine kinase PKB (Burgering and Coffer, 1995). Recent studies have indicated a role for PKB activity in the prevention of apoptosis in a number of systems, including the protection from *c-myc*-induced apoptosis in fibroblasts (Kauffman-Zeh et al., 1997), thus PKB activation may offer an additional mechanism for CD28-mediated protection from apoptosis.

The function of T cell CD80 expression remains largely unknown. The upregulation of both antigen presenting molecules (HLA-DR) and costimulatory molecules (CD80 and CD86) suggests a potential role in the presentation of

antigen, although this may negatively rather than positively regulate T cell responses (Houssaint and Flajnik, 1990; Sidhu et al., 1992; Pichler and Wyss-Coray, 1994; Hargreaves et al., 1997). Clearly the presented findings suggest that one potential function of T cell CD80 may be in the maintenance of T cell survival and the nature and physiological role of CD28-mediated anti-apoptotic signals warrants further investigation. A role for costimulatory signals in T cell survival has been indicated by a number of reports (Groux et al., 1992; Boise et al., 1995; Mueller et al., 1996; Radvanyi et al., 1996; Sperling et al., 1996; Vella et al., 1997) including the demonstration that T cells from CD28 knockout mice effectively initiate proliferation but fail to sustain responses (Lucas et al., 1995) and the recent investigations into the relative provision of costimulatory *versus* apoptosis ligands during antigen presentation (Lu et al., 1997a; Lu et al., 1997b). However, the data presented here appear to be the first demonstration that the provision of CD80 can directly inhibit CD95-mediated apoptosis.

CD80 transfectants and CD86 transfectants were found to be equally effective at inhibiting CD95-mediated apoptosis under these conditions, indicating that either ligand was potentially capable of providing this protective signal. Nevertheless, it would be of interest to determine the relative contribution of CD86 *versus* CD80 in the triggering of CD28-mediated survival signalling under physiological circumstances since these molecules appear to be expressed on APCs with different kinetics (Azuma et al., 1993; Stack et al., 1994; Fleischer et al., 1996). In addition, establishing the duration of the protective signal conferred by CD28 ligation represents a target for future investigation and interestingly work in our laboratory has demonstrated that the provision of CD80 alone (i.e. in the absence of TCR signalling) is sufficient to trigger proliferation in previously activated T cells, but that this response is only intact until approximately 12 days following the initial activation stimulus (Edmead et al., 1996). Thus these data demonstrate the potential for independent signal transduction via CD28, but suggest that this

ability is restricted to a certain time window following the initiation of T cell activation.

Since T cells themselves can clearly express costimulatory ligands in an activation-dependent manner (figure 6.9), it would also be interesting to investigate the potential role of T cell:T cell interactions in the promotion of cell survival *in vivo*. In this regard a recent study has indicated that T cell CD86 molecules may represent preferential ligands for CTLA4 rather than CD28 (Greenfield et al., 1997) and understanding how T cell:T cell interactions may regulate immune responses via apoptosis induction (Hargreaves et al., 1997), anergy induction (Sidhu et al., 1992), CTLA4 signalling (Greenfield et al., 1997) or potentially via CD80/86-mediated survival signalling represents an attractive area of research. These issues are of particular relevance to human T cells given their expression of HLA class II molecules which is not paralleled in mouse T cells (Pichler and Wyss-Coray, 1994). Expression of costimulatory ligands represents a further intriguing difference between mice and humans since CD86 is detectable on resting murine T cells and is subject to downregulation during the activation process whilst conversely CD80 is upregulated (Prabhu Das et al., 1995). This contrasts with the expression patterns observed in human T cells (figure 6.9) and may have a bearing on T cell susceptibility to apoptosis if these ligands were to deliver a similar protective signal in mice.

The finding that the two signals which were required for productive T cell activation were also implicated in conferring resistance to CD95-mediated apoptosis was supported by the supernatant transfer experiments, and highlighted the significance of the *in vitro* activation protocols used for such studies. Clearly suboptimal T cell activation, which may support a degree of short term proliferation but which fails to provide a truly costimulated TCR-derived stimulus, is likely to be defective in the induction of anti-apoptotic signals associated with these pathways. Accordingly, stimulation of purified T cells with

PHA in the absence of ligand for CD28 may be sufficient to trigger suboptimal proliferation, but may not prove effective at protecting cells from apoptosis. Interestingly, one of the studies which is most contentious in the light of my data utilised PHA treatment of purified T cells (i.e. in the absence of CD80 positive APCs) as the initial activation stimulus (Klas et al., 1993), so perhaps the lack of CD95 resistance in these experiments is not surprising since the signals which have been demonstrated in this thesis to confer resistance to apoptosis have not been delivered to these T cells. Similarly in the study by Owen-Schaub and colleagues (Owen-Schaub et al., 1992), purified human T cells were activated simply by the addition of IL-2, a protocol which I view as a form of "bystander" T cell activation since antigen- and costimulation- derived stimuli are lacking, and the sensitivity of these cultures to apoptosis is in line with my data pertaining to T cells stimulated with activated supernatants. The previously presented finding (chapter 3) that "correct" activation of T cells is associated with the upregulation of surface CD28 expression to high levels suggests that CD28 upregulation may represent one potential mechanism for the promotion of survival in cells which have received these activation signals.

One interpretation of these data is that CD80-costimulated SEB, anti-CD3, PHA or ConA responses offer a more physiologically relevant model for T cell activation than does uncostimulated PHA-driven proliferation given our current understanding of the *in vivo* requirements for this process. Rather than representing an isolated finding, the CD95 resistance documented here may therefore accurately reflect the normal *in vivo* situation under conditions when the T cell activation stimulus fulfils a two signal requirement. In addition, these findings are supported by studies on T cells which have been activated using anti-CD3 and anti-CD28 in which resistance to irradiation-induced apoptosis has been observed (Mueller et al., 1996). Moreover, a recent report by Krammer and colleagues documents CD95 resistance in human T cells stimulated with myelin basic protein or tetanus toxoid. Since the CD95-sensitive controls for these

experiments were Jurkat T cell lines (rather than normal activated T cells) it is possible that the CD95 resistance is not specific to these particular antigens, but rather is a feature of "correct" T cell activation involving antigen and costimulatory signals in line with the findings of this thesis.

Since the survival of at least a subset of antigen-specific T cells is presumably a requirement for the maintenance of immune memory, mechanisms must exist to ensure resistance of these T cells to apoptosis, for example following encounter with soluble CD95L. One such mechanism which has been shown to prevent apoptosis in cytokine-deprived T cells involves the secretion of a protective 30kDa soluble factor from stromal cells which is associated with the upregulation of $BCLX_L$ (Gombert et al., 1996). In addition, cytokines which signal through the IL-2 receptor common γ chain can enhance T cell survival via BCL-2 upregulation (Akbar et al., 1993b; Akbar et al., 1996) although paradoxically such interactions may also increase T cell sensitivity to AICD (Wang et al., 1996b). Increased intracellular glutathione levels have also been implicated in the promotion of T cell survival (Hyde et al., 1997). The data presented in this thesis suggest that "correct activation" via signalling through the TCR and CD28 may offer a further mechanism to confer resistance to apoptosis in T cells, in this case in response to CD95 ligation. Clearly T cells must be efficiently eliminated in the wake of an immune response to ensure immune cell homeostasis, however these data indicate that T cells activated by antigen and costimulation are not constitutively sensitive to apoptosis via CD95 ligation and imply that additional interactions, or the termination of protective signalling, may be required for susceptibility to ensue. Alternatively, downregulation of T cell responses may be largely achieved by mechanisms other than CD95 and the growing family of T cell death receptors may be relevant in this regard (Chinnaiyan et al., 1996; Pan et al., 1997).

In summary, the resistance to CD95-mediated apoptosis documented in both chapter 4 and chapter 5 could potentially be conferred by the ligation of receptors such as the TCR and CD28 during the process of productive T cell activation. By linking the nature of the activation process to the delivery of survival signals, this offers a mechanism for allowing cells which may be inappropriately activated, for example bystander T cells, to apoptose following transient activation, whilst cells receiving the requisite signals during priming may be protected from apoptosis via this route. Since the molecules which allow CD95-mediated apoptosis (CD95 and CD95L) are both upregulated during T cell activation, the potential for cell death is already in place at this time and it is therefore likely that T cell survival is manipulated by controlling the signals which confer resistance to apoptosis. The duration of TCR or CD28-mediated resistance to apoptosis has not yet been determined and represents a target for further investigation.

CHAPTER 7

CD95-mediated CD28 Downregulation

7.1 INTRODUCTION

One interesting observation arising from studies on the process of CD95-mediated apoptosis in Jurkat T cells was that CD95 ligation appeared to affect T cell CD28 expression. CD28 is known to provide a potent stimulatory signal to T cells favouring proliferation and cytokine production, and signalling via this receptor has also been implicated in the promotion of cell survival as discussed in the previous chapter. It follows that stimuli which alter the expression levels of this molecule may have important consequences for T cell responses. Therefore experiments were performed to investigate CD95-mediated CD28 downregulation in Jurkat T cells and to document the kinetics and specificity of this response. In addition, the mechanism underlying CD95-mediated CD28 downregulation was investigated.

7.2 RESULTS

During studies on CD95-mediated apoptosis in J16 cells, it became apparent that CD95 ligation could modulate T cell surface receptor expression. Accordingly, treatment with the anti-CD95 antibody CH11 induced a rapid and time dependent decrease in the surface expression of CD28 as measured by surface staining and FACS analysis (figure 7.1). Kinetic analysis demonstrated that loss of CD28 was evident by as little as 3 hours following CH11 treatment, and that by 6 hours the majority of cells exhibited downregulated expression of this receptor (figure 7.1). Downregulation appeared to be specific to CD28 since the levels of other receptors examined, including CD2, CD3, CD5, CD48 and CD58 were not detectably affected by CH11 incubation and data for CD58/LFA3 expression are shown in figure 7.1. In addition a control IgM antibody, OBI, had no detectable affect on the level of CD28 receptor expression (figure 7.1).

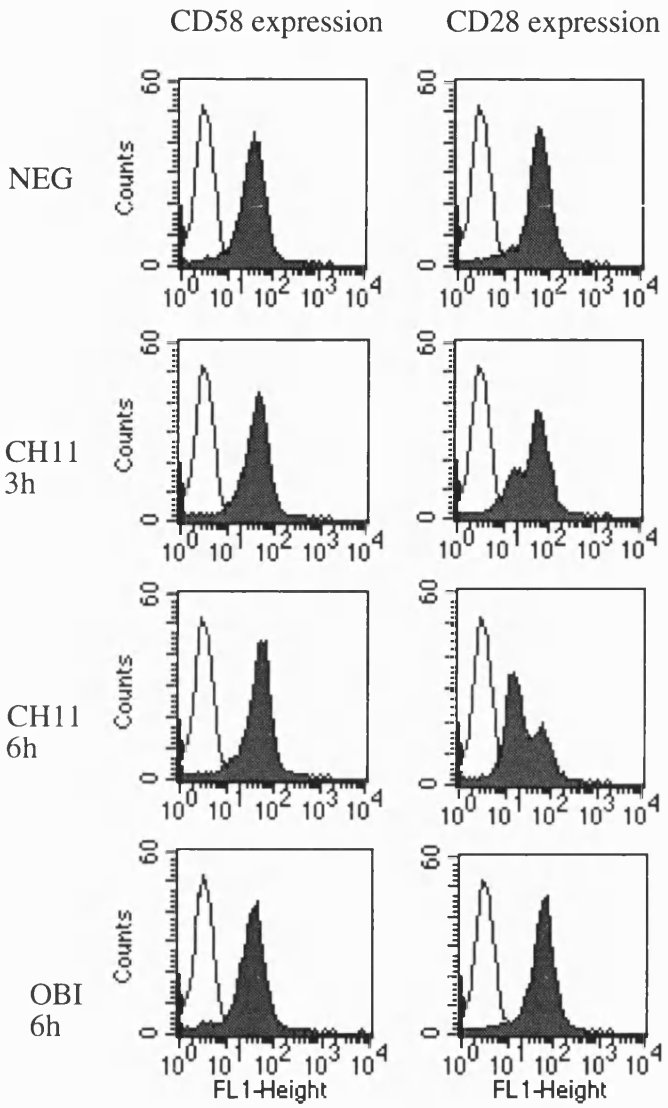


Figure 7.1: Effect of antibody-mediated CD95 engagement on T cell surface marker expression. J16 cells were incubated with anti-CD95 antibody (CH11, 0.05µg/ml) or a control IgM antibody (OBI, supernatant) for the indicated time periods then stained for expression of CD58 or CD28 (filled histograms). Open histograms show staining with secondary antibody only. Data are representative of >8 individual experiments.

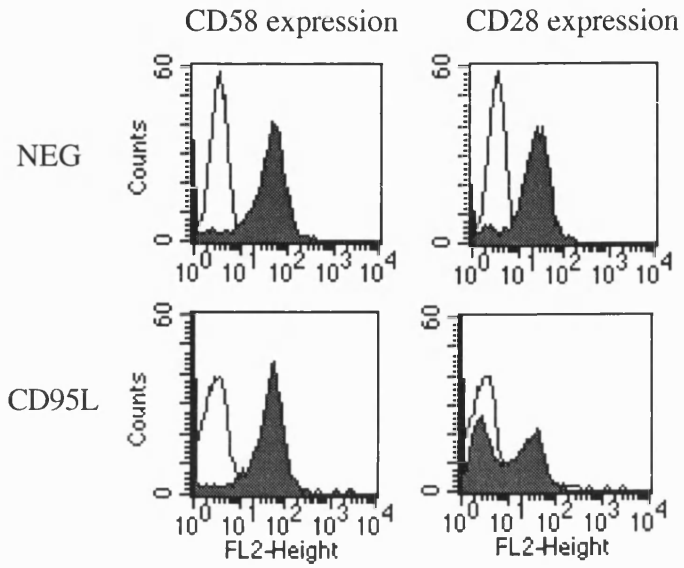


Figure 7.2: Effect of ligand-mediated CD95 engagement on T cell surface marker expression. J16 cells were incubated with control supernatant (NEG) or CD95L (COS-7 cell supernatant) for 4h and stained for surface expression of CD58 or CD28 (filled histograms). Open histograms show staining with secondary antibody only. Data are representative of 3 separate experiments.

	% increase in the CD28 low population relative to control			
	Control	CH11	CH11 + ZB4	ZB4
J16	0	21.75	0.51	-0.36
JLW	0	0.65	ND	ND
SEB blasts	0	11.68	0.1	-1.53

ND = not determined

Table 7.1: Effect of the blocking anti-CD95 antibody ZB4 on CH11-mediated CD28 downregulation. J16 cells JLW cells or SEB blasts (day 6) were incubated for 6h (15h for SEB blasts) with the anti-CD95 antibodies indicated and CD28 expression was measured by FACS. CH11 was used at 0.05μg/ml and ZB4 was used at 0.1μg/ml. The values indicate the % increase in a CD28 low region (arbitory gate) relative to control-treated cells. Data are representative of 3 experiments.

Since antibodies do not necessarily reflect the biological effects of natural ligand, these studies were also extended to the use of soluble CD95L for the provision of CD95 engagement (figure 7.2). In accordance with the CH11 experiments, incubation of J16 cells with soluble CD95L lead to rapid and specific CD28 downregulation without observable differences in the expression of other T cell surface receptors (CD58 expression shown figure 7.2). Thus CD28 downregulation was shown to be a rapid and specific response which occurred following CD95 engagement by both antibody and natural ligand.

In order to elucidate the relationship between CD95-mediated apoptosis induction and CD95-mediated CD28 downregulation, experiments were performed to address whether non apoptotic anti-CD95 antibodies were competent to induce CD28 downregulation. The anti-CD95 antibody ZB4, which binds to CD95 but fails to induce apoptosis did not downregulate CD28 expression (table 7.1) indicating that signal transduction, as opposed to simple antibody-receptor interactions, was a requirement for this effect. Furthermore, CH11-induced CD28 downregulation was inhibited in the presence of ZB4 confirming that this phenomenon is mediated by the CD95 receptor (table 1). This was a useful control since CD28 is known to downregulate following the interaction with its ligand CD80 (chapter 3) thus it was of relevance to preclude the possibility that CH11 was interacting non-specifically with CD28. The tabulated data also indicate that the Jurkat cell line selected for CD95 resistance (JLW) did not appreciably downregulate CD28 expression following CD95 engagement supporting the concept that signal transduction, rather than simple antibody:receptor interactions, was a requirement for this effect (table 1). In addition CD95-mediated CD28 downregulation was observed in SEB activated peripheral blood T cells (table 1) indicating that this response was not confined to transformed cell lines.

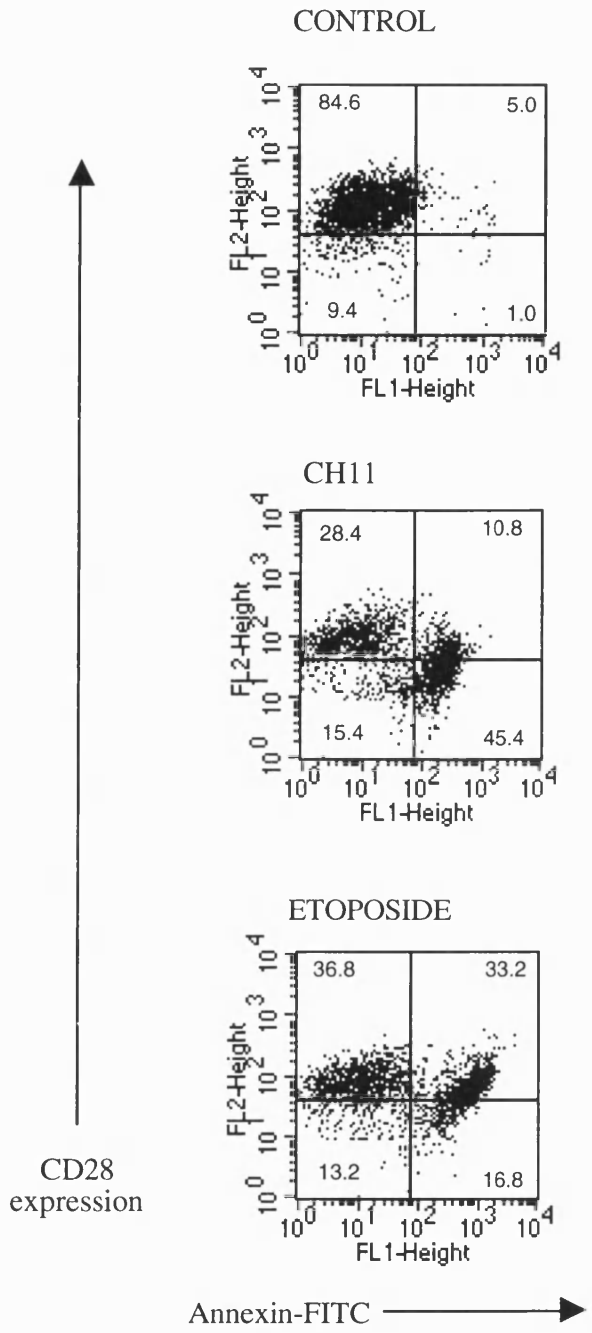


Figure 7.3: Comparison of CD95 ligation and etoposide treatment on T Cell CD28 surface expression. J16 cells were incubated for 6h with the anti-CD95 antibody CH11 (0.05µg/ml) or etoposide (1µg/ml) then stained for surface expression of CD28 and assayed for annexin-FITC binding. Data are representative of 3 similar experiments.

Given that non-apoptotic antibodies did not cause CD28 downregulation, it was therefore of interest to determine whether the cells which had downregulated CD28 following CH11 treatment were committed to apoptosis. Since the annexin-FITC assay detects very early events in the apoptotic pathway and effectively measures commitment to apoptosis (Martin et al., 1995b) this assay was therefore utilised. Dual staining for surface CD28 receptor expression and annexin-FITC binding revealed that those cells which had downregulated CD28 expression following CH11 treatment were in the early apoptotic (annexin bright) population (figure 7.3). It appeared, therefore, that decreased surface expression of CD28 was linked to the induction of apoptotic signalling via the CD95 receptor. Therefore to assess whether downregulation of CD28 was a common feature of apoptosis-inducing signals in T cells, the topoisomerase II inhibitor etoposide was utilised to provide an alternative model of apoptosis induction. Treatment with this compound lead to the appearance of an distinct annexin bright population (44% were induced to express PS under these conditions), however the levels of CD28 expression nevertheless remained high in these cells. These data indicated that CD28 downregulation was not simply a feature of the induction of apoptosis in T cells, but rather was a specific response following CD95 signal transduction.

In an attempt to distinguish the signals required for CD28 downregulation from those necessary for apoptosis induction, a number of experiments were performed. CD95 signal transduction is reported to involve acidic sphingomyelinase activity (Cifone et al., 1993; Gulbins et al., 1995), and one published approach for the inhibition of this enzyme is the use of chloroquine (2mM), a lysosomotrophic agent which neutralises the acidic environment necessary for acidic sphingomyelinase activity (Boucher et al., 1995). In order to assess the role of CD95-induced acidic sphingomyelinase signalling in CD28 downregulation, experiments were undertaken to elucidate the effect of chloroquine on this process. To establish the concentration of chloroquine which effectively inhibited

(A)

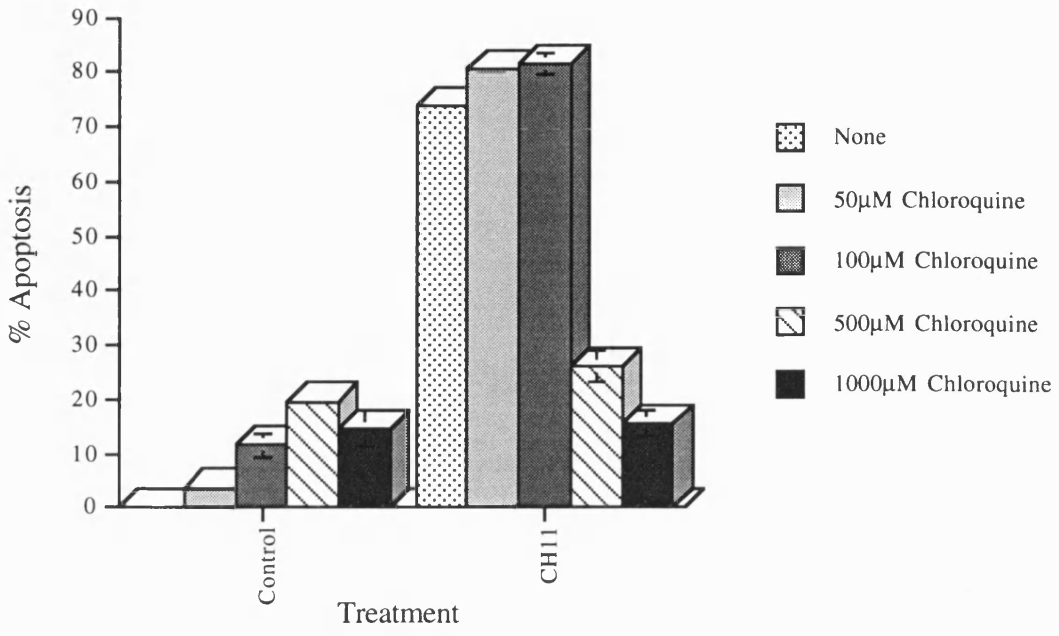


Figure 7.4. Effect of chloroquine on CD95-mediated apoptosis. (A) ^{3}H-thymidine labelled J16 cells were pre-treated for 1h with the indicated concentration of chloroquine prior to addition of the anti-CD95 antibody CH11 (0.1µg/ml) for a further 4h incubation. Apoptosis was measured by JAM assay and is calculated as the % decrease in CPM relative to control-treated J16 cells. Columns represent the mean (+/-SEM) of triplicate wells. (B) Parallel analysis of membrane integrity by PI exclusion was carried out on cells treated with the indicated concentrations of chloroquine as described above. 10^{5} cells were removed for FACS analysis immediately prior to harvesting for JAM analysis. Loss of membrane integrity is visualised as an increase in FL-2 fluorescence due to PI uptake. Data are representative of 3 similar experiments.

(B)

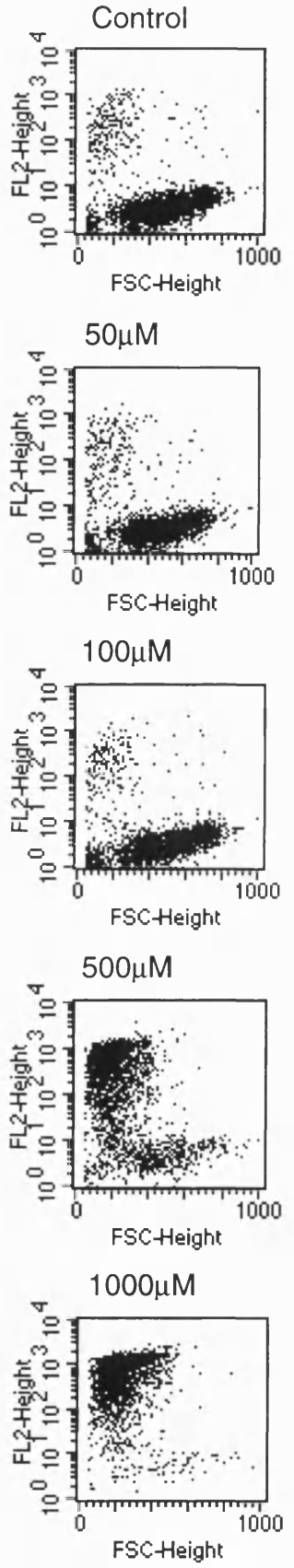


Figure 7.4 cont.

CD95-mediated apoptosis the JAM assay was used, and in order to control for any toxicity resulting from chloroquine treatment, PI exclusion was simultaneously monitored by FACS. Examining the results of the JAM assay in isolation (figure 7.4, panel A), it appeared that chloroquine treatment effectively inhibited CD95-induced apoptosis, since the percent apoptosis following CH11 treatment fell from 73.9% to 26.3% in the presence of this compound (500μM). In addition, the control data indicated that incubation of Jurkats with these chloroquine doses in the absence of CH11 induced only a minor increase in DNA fragmentation as measured by JAM assay. However, when the PI exclusion assay was analysed (figure 7.4, panel B), it became clear that at these doses chloroquine was having a toxic effect resulting in the loss of membrane integrity and consequent PI uptake. Ironically, since the JAM assay is relatively specific to apoptosis detection, as opposed to necrosis measurement, the toxicity of chloroquine was not visualised as cell death in this assay. Similarly, the use of any assay which relies upon characteristics such as DNA fragmentation to measure apoptosis would not detect the necrotic effect of a compound such as chloroquine, and may explain why certain apoptosis inhibition studies yield contradicting results (Eischen et al., 1994; Schraven and Peter, 1995; Janssen et al., 1996). These data emphasise the need for caution in the interpretation of apoptosis inhibition studies since cells which have already undergone necrosis will no longer be able to activate an apoptotic pathway leading to DNA fragmentation: thus treatments resulting in necrotic cell death may be misinterpreted as inhibiting apoptosis induction. Hence, apart from providing the valuable lesson that "dead cells don't apoptose", chloroquine did not appear to be a useful tool for the inhibition of CD95 signal transduction in Jurkat T cells and alternative options were therefore explored.

In addition to acidic sphingomyelinase, CD95 ligation also triggers the activation of a cascade of cysteine proteases (caspases) which are believed to be essential for the successful induction of apoptotic cell death in T cells. Despite the identification of a number of putative substrates for these enzymes including β-

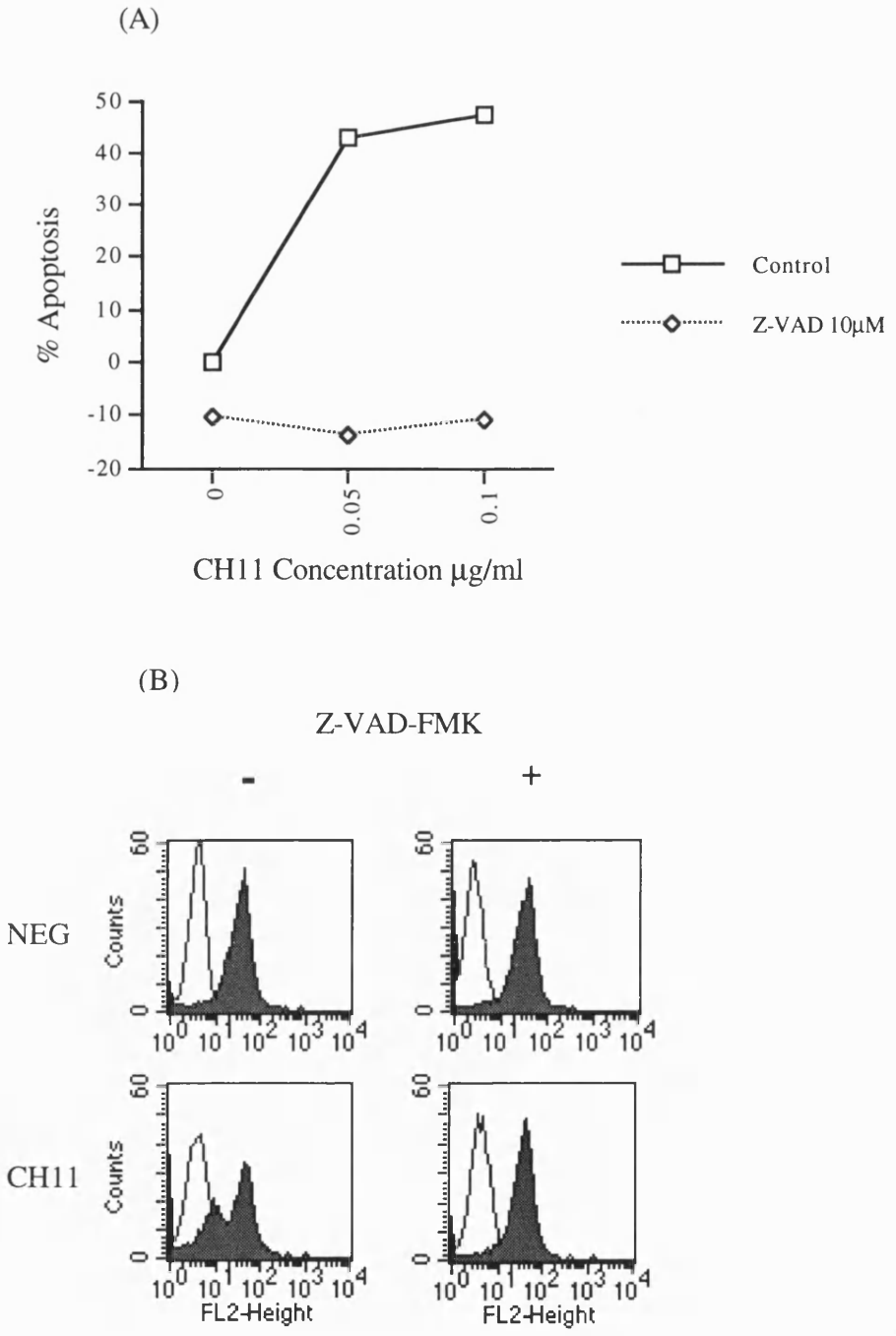


Figure 7.5: Effect of the caspase inhibitor Z-VAD-FMK on CD95-mediated apoptosis and CD28 downregulation. (A) ^{3}H-thymidine labelled J16 cells were pre-incubated for 1h with Z-VAD-FMK (10µM) then treated for 4h with the indicated concentration of the anti-CD95 antibody CH11. Apoptosis was measured by JAM assay and is presented as the % apoptosis relative to control-treated samples The mean (+/-SEM) of triplicate wells is shown. (B) J16 cells were pre-incubated for 1h with 10µM Z-VAD-FMK then treated for 4h with CH11 (0.05µg/ml) prior to staining for CD28 expression (filled histograms). Open histograms indicate staining with secondary antibody alone. Data are representative of 3 experiments.

actin, α-fodrin and lamin B1 (Lazebnik et al., 1994; Martin et al., 1995a; Martin and Green, 1995), it is not yet clear which caspase target is responsible for committing cells to apoptosis. However, a requirement for caspase activity for apoptosis induction can be demonstrated with the use of cell permeant peptide fluoromethyl ketones such as Z-VAD-FMK (Cbz-Val-Ala-Asp(OMe)-fluoromethyl ketone) which inhibits a broad range of caspases (Sarin et al., 1996). Treatment of J16 cells with this caspase inhibitor potently inhibited CD95-mediated apoptosis induction in the JAM assay, as illustrated in figure 7.5 (A). To assess whether caspase inhibition was a useful tool for the segregation of the signals required for apoptosis induction from those required for CD28 modulation, the effect of Z-VAD-FMK on CD95-induced CD28 downregulation was also examined. The data presented in figure 7.5 (B) illustrate that Z-VAD-FMK was in fact an effective inhibitor of CD95-induced CD28 downregulation, demonstrating that the signals which mediate the change in CD28 expression are downstream of a caspase-driven step. It was therefore not possible with the available reagents to separate CD28 downregulation from apoptosis induction.

In order to further characterise CD95-mediated CD28 downregulation, studies were undertaken to determine the mechanism by which this process operated. Surface receptor expression can be modulated in a variety of ways, including receptor internalisation, receptor shedding or even changes in the production or stability of the mRNA which encodes that receptor. To address the latter possibility, experiments were performed to determine whether CH11 treatment resulted in a difference in the detectable levels of CD28 mRNA. Total cellular RNA was therefore extracted from control- and CH11-treated J16 cells and northern blot analysis performed. Methylene blue staining of the RNA following transfer to a nylon membrane allowed the ribosomal bands to be visualised (figure 7.6, panel C) and indicated loading equivalence: RNA from CHO cells was included as a negative control for CD28 and LFA3 expression. Hybridisation studies with a CD28-specific probe revealed a striking reduction in CD28 mRNA

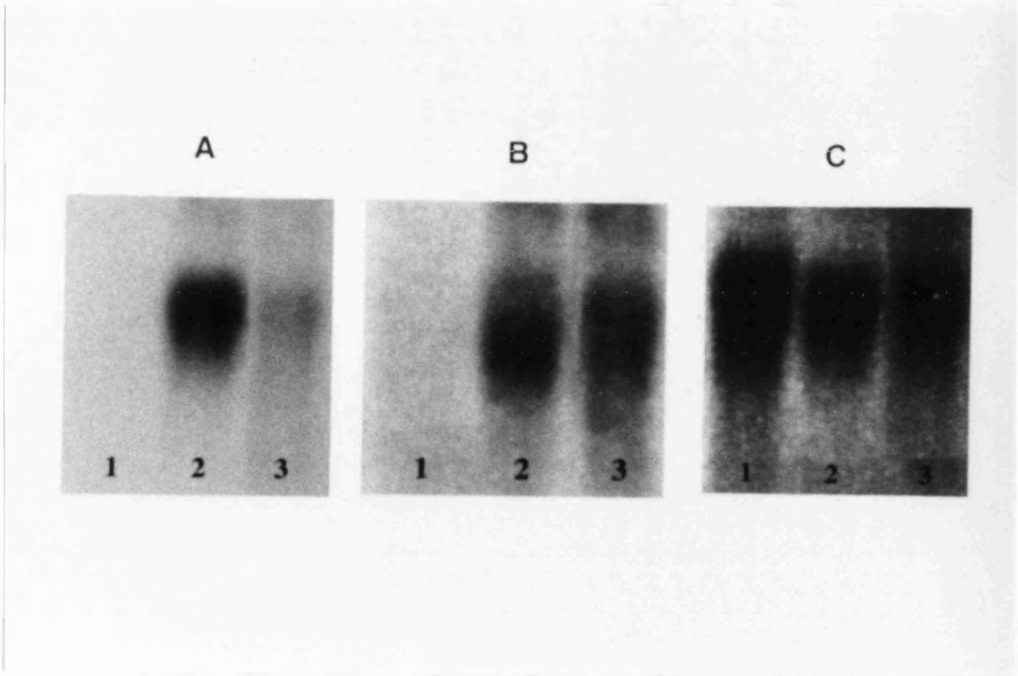
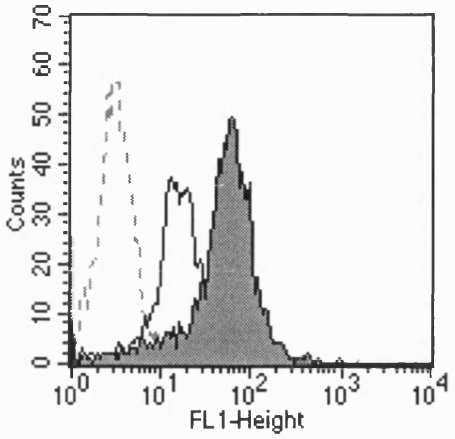


Figure 7.6: Effect of CD95 engagement on CD28 mRNA levels. J16 cells were treated for 4h in the presence of absence of the anti-CD95 antibody CH11 (0.05μg/ml). RNA was isolated and probed for CD28 (panel A) or CD58 (panel B). Binding of ^{32}P-labelled probes was visualised by autoradiography. CHO cells were used as a negative control since they do not express the T cell molecules CD28 or CD58. Total RNA was visualised by methylene blue staining (panel C). Lane 1 = CHO. Lane 2 = untreated J16 cells. Lane 3 = CH11-treated J16 cells.

(A) CH11



(B) CD80

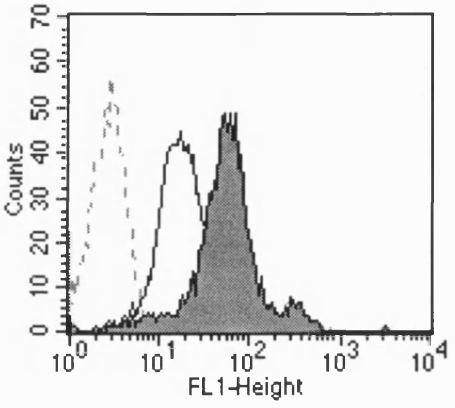


Figure 7.7: Comparison of CH11- and CD80-induced CD28 downregulation. J16 cells were treated with the anti-CD95 antibody CH11 (0.05µg/ml) or CD80 transfectants (at a ratio of 1:3, transfectant:T cell) for 7h then stained for surface expression of CD28. Transfectants were gated out on autofluorescence during FACS analysis. The filled histogram shows CD28 expression on untreated cells and the solid line represents treated cells. The dotted line shows control staining with secondary antibody alone. Data are representative of 3 experiments.

levels as early as 4 hours following CH11 treatment (figure 7.6, panel A). The efficiency of recovery of RNA from both control and CH11-treated cells was equivalent at this time point as determined by spectrophotometric analysis suggesting that there was no generalised degradation of RNA at this time. Furthermore, probing for CD58 revealed no obvious decrease in the mRNA for this protein following CH11 treatment (figure 7.6, panel B). These data therefore indicated that the effect of CD95 ligation on CD28 surface expression was operating, at least in part, at the level of transcription.

The downregulation of CD28 in peripheral blood T cells following engagement with its own ligand (CD80/86) has already been presented (chapter 3) and further studies were therefore carried out to investigate whether this response also occurred in Jurkat cells and to compare it with CD95-mediated CD28 downregulation. These data revealed that CD80-mediated CD28 downregulation was conserved in Jurkat T cells indicating similarity in the regulation of CD28 expression between Jurkats and normal peripheral blood T cells (figure 7.7). In addition, this experiment showed that the processes of CD95-mediated and CD80-mediated CD28 downregulation occurred with broadly similar kinetics since downregulation was largely complete by 7 hours with either treatment (figure 7.7).

7.3 DISCUSSION

The initial observation that CD95 derived signals could mediate T cell surface receptor modulation proved to be a phenomenon which was specific to the CD28 receptor and which was triggered following both antibody and natural ligand (CD95L) treatment. The rapidity and specificity of this response was suggestive of potential biological relevance, as opposed to representing merely a side effect

of apoptosis induction, and this finding was therefore subject to further exploration.

In an attempt to dissect which CD95-induced signals were required for CD28 downregulation and which were required for apoptosis induction, a number of experiments were performed. Initial studies with chloroquine to inhibit acidic sphingomyelinase revealed that toxicity was associated with this compound when used at the concentrations deemed to inhibit apoptosis, and demonstrated the caution required for the use of pharmacological inhibitors in apoptosis assays since such experiments must also control for necrotic cell death. The caspase inhibitor Z-VAD-FMK was therefore utilised as an inhibitor of CD95 signalling since this reagent did not induce toxicity as measured by PI incorporation (in fact it was previously demonstrated to inhibit the loss of plasma membrane integrity associated with long term CH11 incubation in figure 3.1, panel A). At the time when these experiments were performed, the activation of caspases was thought to be a late event in CD95-mediated signal transduction, occurring immediately prior to cell death and thus representing commitment to an apoptotic fate. Therefore, by using this reagent it was envisaged that the CD95 pathway would be inhibited at a distal point such that the ultimate induction of death would be prevented, but the upstream (membrane proximal) components of CD95 signalling would be allowed to proceed. Thus whilst cell death would be prevented under these conditions, the CD95-derived signals which controlled CD28 downregulation might remain intact. The consequent demonstration that CD28 downregulation could occur in the absence of apoptosis induction would negate the possibility that CD28 downregulation was a result of cell death. However, as indicated in figure 7.5 (B) Z-VAD-FMK did in fact inhibit CD95-mediated CD28 downregulation and therefore could not be used to segregate the signalling requirements for CD28 downregulation and apoptosis in this manner.

Subsequent studies on the role of caspase signalling have indicated that the positioning of caspase activity just prior to cell death is an oversimplification. The temporal hierarchy of caspases in the apoptotic pathway is now beginning to be unravelled and current data suggest that whilst caspase 1 activation is a relatively early event, occurring prior to mitochondrial permeability transition, caspase 3 activation lies further downstream in this pathway, and appears to occur after these mitochondrial changes (Susin et al., 1997). Accordingly, the activation of caspase 1 does not appear to represent commitment to apoptosis since the induction of cell death can be prevented downstream of this event, for example by $BCLX_L$ (Boise and Thompson, 1997). Therefore, if the signals required for CD28 downregulation were mediated by caspase 1, then Z-VAD-FMK could inhibit both CD28 downregulation and apoptosis, yet under physiological conditions CD28 downregulation could potentially occur without apoptosis induction in the presence of $BCLX_L$.

Furthermore, during the course of these studies a caspase which associates directly with the cytoplasmic tail of the CD95 receptor was identified (caspase 8) (Muzio et al., 1996; Medema et al., 1997) implicating caspase activation as an even earlier event in CD95-mediated signal transduction than previously considered. The additional finding that caspase inhibitors can block CD95-induced ceramide generation in Jurkat cells (Gamen et al., 1996) places caspase activity upstream of sphingomyelinase activation in this system. Thus as a result of the limited number of tools available as inhibitors of CD95 signal transduction, it was not possible to segregate the signals required for CD28 downregulation from those necessary for apoptosis induction since caspase activation is clearly an early signal which precedes both events. However, the fact that CD28 downregulation is not a feature of etoposide-induced apoptosis provides compelling evidence that this phenomenon is not a result of apoptosis induction, but rather is a specific result of CD95 signal transduction.

Perhaps the most interesting finding pertaining to these studies on CD95-mediated CD28 downregulation, was the mechanistic data which suggested that CD95 signalling induced a dramatic decrease in mRNA for CD28. The levels of mRNA for other proteins (CD58 shown in figure 7.6) did not appear to be affected under these conditions whilst in contrast CD28 mRNA was almost undetectable only 4 hours following CH11 treatment. The fact that the observed downregulation appeared to be transcriptionally controlled suggested that the CD28 molecule could be specifically targeted in this way, allowing CD28 functions to be rapidly silenced under certain circumstances. Furthermore, the fact that a transcriptional event is so rapidly perceived at the protein level indicates that the duration of CD28 molecules at the cell surface is relatively short, again implying that surface expression of this receptor is carefully controlled. Consistent with this supposition, data have already been presented illustrating the tight control of CD28 expression during the T cell activation process, as surface expression of this receptor is both downmodulated by CD80 engagement and subsequently upregulated to high levels (chapter 3).

Intriguingly, the downregulation of CD28 following engagement with its own ligand (CD80/86) has also been reported to be under transcriptional control (Linsley et al., 1993), and the decrease in receptor expression occurs with similar kinetics to that seen following CD95 engagement (figure 7.7). Whilst CD95 and CD28 are historically believed to have widely different biological functions, that of apoptosis induction *versus* that of costimulation, they nevertheless appear to posses certain functional similarities. For example, CD95 ligation has been reported to costimulate T cell proliferation under certain circumstances (Alderson et al., 1993) suggesting that CD95 signalling can potentially provide components of a CD28-like pathway. In this regard, CD95 shares a number of signalling features with CD28 including the activation of sphingomyelinase (Boucher et al., 1995; Tsuruta et al., 1995; Edmead et al., 1996) and JNK (Su et al., 1994; Wilson et al., 1996). Since both CD80 and CD95L induce CD28 downregulation, and

that in both cases the process appears to be transcriptionally controlled, it is possible that CD28 downregulation is a consequence of a common signalling activity following the crosslinking of either CD28 or CD95, as indicated in figure 7.8.

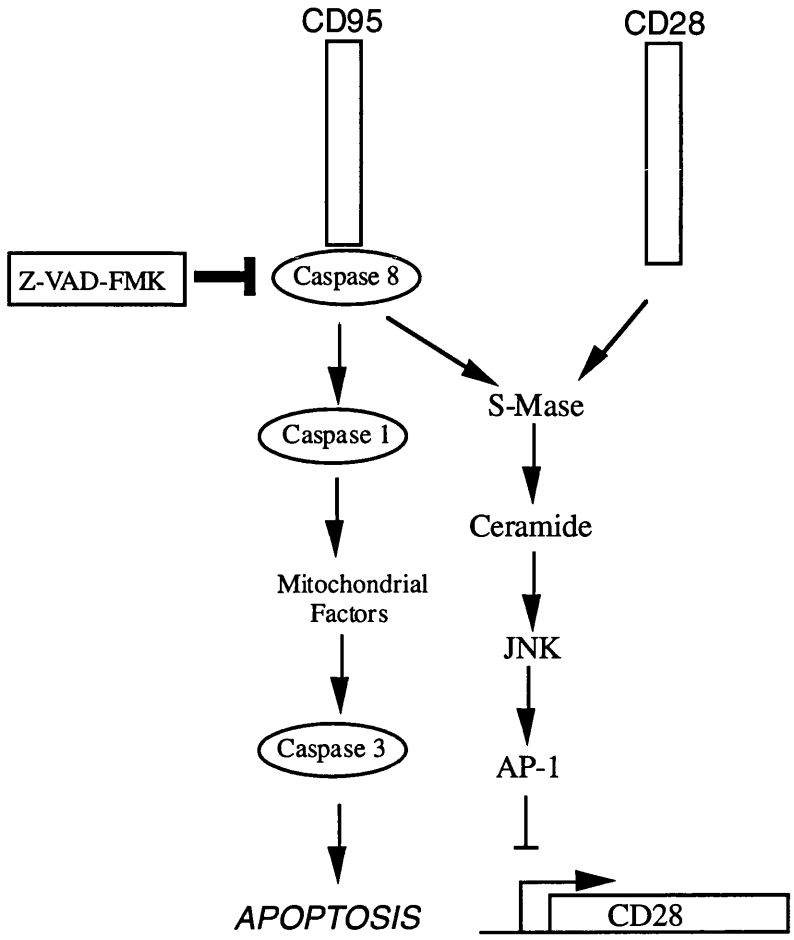


Figure 7.8: Schematic diagram illustrating both shared and distinct components within CD95 and CD28 signalling pathways. Since the sphingomyelinase pathway is common to both receptors, this represents a candidate pathway in the CD95- and CD28-mediated downregulation of CD28 mRNA. S-Mase = sphingomyelinase, JNK = c-Jun N-terminal kinase, AP-1 = Adaptor Protein 1.

Given the involvement of acidic sphingomyelinase in both CD28 and CD95 signalling, one candidate signalling intermediate involved in CD28 surface modulation is ceramide. Ceramide is implicated in diverse biological processes

including proliferation, growth arrest and apoptosis (Boucher et al., 1995; Jayadev et al., 1995; Tepper et al., 1995) all of which are consistent with the activation of this pathway by both CD28 and CD95. In addition, ceramide generation has been linked to the triggering of both NFκB and JNK, and work by colleagues in this laboratory has recently established that whilst CD28 can activate both pathways in normal T cells (Edmead et al., 1996), CD95 triggers only JNK activation. Thus ceramide-induced JNK is common to both CD28 and CD95 pathways and represents a potential candidate for negatively regulating the transcription of the CD28 gene as illustrated in figure 7.8, presumably via the transcription factor AP-1 of which it phosphorylates the c-Jun component (Su et al., 1994).

The phenomenon of CD95-mediated CD28 downregulation may be of interest in the context of HIV infection where T cells have been shown to express diminishing levels of CD28 with disease progression (Choremipapadopoulou et al., 1994) and intriguingly exhibit increased sensitivity to CD95-mediated apoptosis (Estaquier et al., 1995). One implication of these findings is that CD28 downregulation may promote CD95 killing, possibly contributing to the CD4+ T cell depletion associated with AIDS. Thus T cell CD28 expression may be providing a protective mechanism which is gradually lost during HIV infection. Further support for this concept comes from the demonstration that CD28 expression is downregulated in late passage T cell clones and that this phenotype is associated with increased susceptibility to apoptosis (Pawelec et al., 1996).

In summary, therefore, these data link the two key T cell receptors CD28 and CD95 by illustrating that CD95 ligation leads to rapid and specific downregulation of CD28 expression. This phenomenon appears to be under transcriptional control and is associated with the induction of early apoptotic membrane changes (PS exposure). The fact that both CD80 and CD95L can trigger the downregulation of CD28 at a transcriptional and protein level is indicative of functional similarities between the signalling pathways initiated by

CD28 and CD95, and further work is required to identify which component(s) of these pathways is responsible for mediating CD28 downregulation. Both CD80-mediated and CD95L-mediated CD28 downregulation also occur in normal peripheral blood T cells, and therefore represent relevant targets for further study.

CHAPTER 8

Conclusions

The T cell response to antigen engagement is subject to considerable regulation depending on the context in which TCR signalling is initiated. For example, CD28 costimulation extensively modifies the outcome of antigen presentation in terms of ^{3}H-thymidine incorporation (Gimmi et al., 1991; Linsley et al., 1991a; Razi-Wolf et al., 1992; Sansom et al., 1993), cytokine production (Lindsten et al., 1989; Fraser et al., 1991; Jenkins et al., 1991; Fraser and Weiss, 1992; Thompson et al., 1993) and the promotion of T cell survival (Groux et al., 1992; Radvanyi et al., 1996). Whilst CD28 positively regulates T cell activation, conversely, engagement of its homologue, CTLA4, provides a negative regulatory signal (Walunas et al., 1994; Krummel and Allison, 1995; Krummel and Allison, 1996; Walunas et al., 1996). In addition, the availability of ligands for death receptors such as CD95 may influence the nature or magnitude of the subsequent T cell response (Lu et al., 1997a; Lu et al., 1997b). Thus the net result of T cell antigen recognition is determined by multiple receptor:ligand interactions which collectively allow T cell responses to be regulated in the periphery. Consequently such mechanisms form the basis for discrimination between the generation of tolerance and immunity following the encounter of peripheral T cells with antigen. Data from knockout mice imply that peripheral tolerance mechanisms operated by receptors such as CTLA4 and CD95 may be equally important in the prevention of autoimmunity as central tolerance by thymic deletion (Bluestone, 1997). The lymphoproliferation and autoimmune features that characterise mice deficient in CD95 (MRL-*lpr*) or CD95L (MRL-*gld*) reveal a key role for the CD95 system in the maintenance of peripheral tolerance, presumably via the induction of apoptosis to preclude and/or terminate immune responses. However, the specific contribution of T cells to this phenotype remains unclear and the relative importance of CD95-mediated elimination of B cells, and APCs in the prevention of autoimmunity is poorly understood. Evidence from *in vivo* (Ettinger et al., 1995) and *in vitro* (Dhein et al., 1995; Boshell et al., 1996) work has demonstrated the involvement of CD95-induced T cell apoptosis in the downregulation of T cell responses to superantigen, supporting a role for CD95 in the maintenance of

immune cell homeostasis. Nevertheless, an appreciation of the mechanistic and kinetic regulation of the CD95 system, particularly in humans, is lacking and formed the basis for this investigation.

This study therefore examined the expression and function of CD95 and CD95L in the context of defined *in vitro* activation conditions in order to elucidate the role of the CD95 system in the control of human T cell responses. Results indicate that, contrary to current perceptions, activated human T cells are largely resistant to CD95-mediated apoptosis despite upregulation of CD95, and activation-induced expression of CD95L. Thus these data provide a new perspective on the role of the CD95 pathway both in the killing of T cells by interaction with CD95L on a neighbouring cell, and the triggering of T cell suicide by AICD following TCR restimulation. Since the findings appear controversial in the light of work by Klas and colleagues (Klas et al., 1993), a large proportion of the study has rigorously analysed the systems used in order to verify that the observed resistance to apoptosis was not artefactual. Carefully controlled experiments have consistently documented CD95 resistance and have suggested that the discrepancy with the above study is more likely to arise from the unusual T cell activation conditions employed in which the provision of costimulation was lacking. Since the data in this thesis indicate that protection from CD95-mediated apoptosis is associated with the provision of TCR and CD28 signalling, apoptosis sensitivity in T cells stimulated with PHA in the absence of costimulatory ligand is not entirely unexpected. Indeed, we have been able to demonstrate that the provision of costimulation during PHA activation of purified T cells can convert CD95 sensitivity to CD95 resistance (McLeod et al., 1997).

It is pertinent to question how the dogma that activated human T cells were CD95 sensitive came to be so widely accepted on the basis of so few reports. As previously highlighted (chapter 4), the percentage CD95-induced apoptosis in these studies is not always dramatically higher than that documented here, yet the

conclusions drawn are substantially different. Thus there may have been a tendency to interpret data from human T cells such that it was consistent with the prevailing hypothesis that CD95-expressing cells were CD95 sensitive. Whilst clearly this is the case for a proportion of activated human T cells, the data presented here suggest that in fact resistance to apoptosis may predominate in these cultures, implying the existence of protective mechanisms in most, but intriguingly not all, of these cells.

Current thinking regarding CD95-mediated apoptosis has largely been influenced by studies on murine T cells (Brunner et al., 1995; Ju et al., 1995; Ettinger et al., 1995) with little concession to the potential differences which may exist in the human system. Since there are clearly cell type-specific differences in the mechanism by which CD95 induces apoptosis, illustrated for example by the requirement for elevated intracellular calcium for CD95 signalling in human B cells (Oshimi and Miyazaki, 1995) but not T cells (Gulbins et al., 1995; Vignaux et al., 1995), it seems likely that species-specific differences may also exist. Differential control of peripheral tolerance mechanisms in mice and humans is not altogether a surprising finding given the evolutionary divergence and thus the differing immune requirements of these two species. Accordingly, whilst a lack of primary health care makes the successful induction of immunity a necessity in the murine system, the short life span associated with this species renders the prevention of autoimmunity potentially less of a priority, since tolerance must only be maintained for a relatively short time period. In contrast, the markedly increased longevity typical of humans demands the existence of long term tolerance mechanisms and conversely the maintenance of immune memory is likely to be altered in humans for similar reasons. Clearly there are many parallels between mice and humans in terms of the molecules involved in peripheral tolerance and much can be learnt from *in vivo* studies in this area, however subtle inter-species differences in immune features serve as a timely reminder that the functional biology of mice and humans cannot be assumed to be identical. Most

notably the lack of MHC class II expression (the murine equivalent of HLA molecules) on activated murine T cells presumably precludes the possibility of T cell:T cell antigen presentation in this species. Conversely, the induction of HLA class II molecules on human T cells permits such interactions and may offer an additional mechanism to negatively regulate T cell responses (Houssaint and Flajnik, 1990; Sidhu et al., 1992; Pichler and Wyss-Coray, 1994; Hargreaves et al., 1997).

Hypothesis: A Personal Perspective on the CD95 System

In seeking to understand the role of the CD95 system in the human T cell response, it is relevant to consider why and when we need activated T cells in order to mount a successful defence response. For what purposes do we require clonal T cell expansion? When does this response need to be shut down? What is the role of CD95 in respectively limiting or mediating these processes? Specific antigen-reactive T cells are clonally expanded in order to a) facilitate antibody production from B lymphocytes which share the same antigen specificity (although may recognise a different epitope of that antigen) b) perform cytotoxic functions and c) co-ordinate the recruitment and activation of additional immune cells including accessory cells (neutrophils, basophils, eosinophils) and macrophages. The requirements of an effective immune system are to ensure that defence responses are mounted against foreign antigens but not self tissue, and that subsequently the contributory immune cells are eliminated or silenced such that the *status quo* is restored yet immune memory is maintained. Consequently the control of T cell activation and the ultimate demise of T cells by apoptosis must reflect these aims.

In contemplating the involvement of CD95 in the above processes, it is tempting to presume that CD95-mediated apoptosis functions in the latter stages of an

immune response to facilitate the removal of activated T cells. Such a prediction is consistent with the activation induced upregulation of both CD95 and CD95L and a situation in which T cells could be eliminated by cell suicide (AICD) or by interaction with CD95L-expressing APCs. Since the pathogen responsible for initiating the immune response should have been effectively removed by this stage, the expression of costimulatory ligands on APCs may now decrease given that pathogen-associated products (such as LPS) are implicated in the upregulation of these molecules (Razi-Wolf et al., 1992). Thus there may be preferential expression of death ligands such as CD95L as opposed to costimulatory ligands (CD80/86) on APCs at this time, a situation which could potentially favour T cell apoptosis rather than T cell activation in line with published studies (Lu et al., 1997a; Lu et al., 1997b) and consistent with the potentiation of CD95-mediated apoptosis by inclusion of CTLA4-Ig (figure 6.10).

Whilst the downregulation of T cell responses is clearly important and apoptosis is a likely mechanism for achieving this, the timing of this process must nevertheless be consistent with the fulfilment of T cell immune function. For example, the CD95-induced elimination of T cells only four or six days following stimulation consistent with the 2 reports previously cited (Owen-Schaub et al., 1992; Klas et al., 1993), is difficult to reconcile with a role for such cells in the provision of effective B cell help or cytotoxic function. Given the observed kinetics of serum antibody detection and isotype switching (also believed to be T cell dependent), and the timing of peak T cell responses (MacDonald et al., 1991; MacLennan et al., 1997), acquisition of cytotoxic function (Ortiz et al., 1997) and expression of VLA antigens (Ortiz et al., 1997), it is clear that survival of at least some T cells beyond day six may be necessary for an effective immune response to be mounted. In addition, it is envisaged that at least a subset of antigen-specific T cells must persist to provide immune memory such that secondary immune responses can be rapidly initiated upon re-infection. Thus in postulating a role for CD95-mediated apoptosis in the termination of an immune response, the timing of

the T cell contribution to immunity should be considered: if the only limit on CD95 sensitivity was a four to six day maturation period, it is difficult to see how this is consistent with the effective provision of immune function. Whilst the CD95 system may contribute to the termination of immune responses, there is also likely to be an important role for growth factor deprivation in the induction of apoptosis under these circumstances. In particular, since the initiating pathogen may have been effectively eliminated by this stage, there is likely to be reduced antigen availability for the triggering of AICD, consistent with the possibility that cytokine deprivation induced apoptosis may be a key mechanism for removal of T cells at these times (Akbar et al., 1993a; Akbar et al., 1996).

The data presented in this thesis support an alternative (although not mutually exclusive) role for the CD95 system in the differential apoptosis of bystander versus "correctly activated" T cells. Results demonstrate that T cells which have been activated in the presence of ligand for TCR and CD28 are largely protected from CD95-mediated apoptosis, and that the triggering of TCR or CD28 signals in T blasts can promote resistance to CD95. The proportion of the population exhibiting CD95 sensitivity in these studies (up to approximately 35%) may represent T cells which did not receive appropriate activation signals but instead may have become activated in a "bystander" manner due to the cytokine-rich environment. Since CD95 expression is relatively homogeneous in these cultures and the transfer of supernatant from activated blasts to resting T cells upregulates CD95 expression, this suggests that bystander activated T cells can express equivalent CD95 to those which were correctly activated by antigen and costimulation. Consistent with the hypothesis that CD95 sensitive cells may have lacked one of these two signals, this subset is characterised by low CD28 expression (figure 6.7) whereas the co-ordinated provision of both TCR and CD28 signalling is associated with the upregulation of CD28 expression to high levels (figures 3.8-3.9).

Thus a key role for the CD95 system may be in limiting the expansion of bystander activated T cells rather than solely in the termination of immune responses. Accordingly T cells activated non-specifically by the cytokine environment may initiate a proliferative response, but the subsequent rapid acquisition of CD95 sensitivity in such cells, consistent with the findings of Klas and colleagues (Klas et al., 1993) may limit their ability to drive an effective immune response. The expression of functional CD95L on correctly activated T cells, as documented in this thesis, would provide a mechanism for the removal of CD95 sensitive bystander T cells, in addition to the deletion of autoreactive B cells as previously reported (Rathmell et al., 1995). Furthermore, the release of soluble CD95L would preclude the requirement for cell:cell contact in the elimination of these potentially dangerous immune cell subsets. A role for CD95 early on in the prevention of immune response initiation (rather than solely in immune response termination) is more commensurate with the kinetics by which CD95 sensitivity is acquired in the studies by Klas and colleagues and Owen-Schaub and colleagues (Owen-Schaub et al., 1992; Klas et al., 1993), and is supported by the detectable effect of CD95 stimulation during the initial stimulation of resting T cells in three day proliferation assays (figure 3.4). Such a model is consistent with the requirement for functional CD95 and CD95L in the maintenance of peripheral tolerance, as indicated by the autoimmune phenotype of MRL-*lpr* and -*gld* mice, since bystander T cells by definition have bypassed the two-signal activation requirements designed to preclude responsiveness against self tissue. Thus any defect in the CD95 system is likely to compromise the deletion of potentially autoreactive T cells and consequently may allow the onset of autoimmunity.

The CD95 resistance documented in this study has prompted a re-assessment of the role of the CD95-mediated apoptosis in the human T cell immune system. In the light of this data, I propose that the CD95 system may exist to regulate the nature rather than the magnitude of immune responses by controlling which T

cells are permitted to survive long enough to provide effector functions, based on the differential apoptosis sensitivity of correctly activated versus bystander T cells. This model is consistent with a role for CD95 in the maintenance of peripheral tolerance by the prevention of bystander-driven autoimmune responses. Thus given the questions raised by this thesis, it is clear that much remains to be learnt about the role of CD95 in T cell homeostasis. A better understanding of how the CD95 system influences the generation of T cell tolerance *versus* immunity will be critical to the manipulation of these outcomes for clinical benefit.

BIBLIOGRAPHY

Ahmed, R. and Gray, D. (1996). Immunological memory and protective immunity - understanding their relation. Science *272*, 54-60.

Akbar, A.N., Borthwick, N., Salmon, M., Gombert, W., Bofill, M., Shamsadeen, N., Pilling, D., Pett, S., Grundy, J.E., and Janossy, G. (1993a). The significance of low BCL-2 expression by CD45RO-T-cells in normal individuals and patients with acute viral-infections - the role of apoptosis in T-cell memory. J. Exp. Med. *178*, 427-438.

Akbar, A.N., Salmon, M., Savill, J., and Janossy, G. (1993b). A possible role for BCL-2 in regulating T-cell memory - a balancing act between cell-death and survival. Immunol. Today *14*, 526-532.

Akbar, A.N., Borthwick, N.J., Wickremasinghe, R.G., Panayiotidis, P., Pilling, D., Bofill, M., Krajewski, S., Reed, J.C., and Salmon, M. (1996b). Interleukin-2 receptor common γ-chain signalling cytokines regulate activated T-cell apoptosis in response to growth-factor withdrawal - selective induction of anti-apoptotic (BCL-2, BCL-X_L) but not pro-apoptotic (BAX, BCL-X_S) gene-expression. Eur. J. Immunol. *26*, 294-299.

Alderson, M.R., Armitage, R.J., Marakovsky, E., Tough, T., Roux, E., Schooley, K., Ramsdell, F., and Lynch, D.H (1993). Fas tranduces activation signals in normal human T lymphocytes. J. Exp. Med. *178*, 2231-2235.

Alderson, M.R., Tough, T., Davis-smith, T., Braddy, S., Falk, B., Schooley, K.A., Goodwin, R., Smith, C.A., Ramsdell, F., and Lynch, D.H. (1995). Fas ligand mediates activation-induced cell death in human T lymphocytes. J. Exp. Med. *181*, 71-77.

Alegre, M-L., Noel, P.J., Eisfelder, B.J., Chuang, E., Clark, M.R., Reiner, S.L., and Thompson, C.B. (1996). Regulation of surface and intracellular expression of CTLA-4 on mouse T cells. J. Immunol. *157*, 4762-4770.

Allen, D.L., Albrecht, D.E., Spencer, M.J., Tidball, J.G., and Edgerton, V.R. (1995). Serum deprivation results in apoptotic cell-death of l6 and C2C12 skeletal myoblasts in-vitro. Mol. Biol. Cell *6*, 2057.

Allen, J.E. and Maizels, R.M. (1997). Th1-Th2: reliable paradigm or dangerous dogma? Immunol. Today *18*, 387-392.

Anderson, M.S. and Miller, J. (1992). Invariant chain can function as a chaperone protein for class-II major histocompatibility complex-molecules. Proc. Natl. Acad. Sci. U.S.A. *89*, 2282-2286.

Anel, A., Buferne, M., Boyer, C., Schmitt-Verhulst, A-M., and Golstein, P. (1994). T cell receptor-induced Fas-ligand expression in cytotoxic T lymphocyte clones is blocked by protein tyrosine kinase inhibitors and cyclosporin A. Eur. J. Immunol. *24*, 2469-2476.

Anel, A., Gamen, S., Alava, M.A., SchmittVerhulst, A.M., Pineiro, A., and Naval, J. (1997). Inhibition of CPP32-like proteases prevents granzyme B- and Fas-, but not granzyme A-based cytotoxicity exerted by CTL clones. J. Immunol. *158*, 1999-2006.

Arase, H., Arase, N., and Saito, T. (1995). Fas-mediated cytotoxicity by freshly isolated natural-killer-cells. J. Exp. Med. *181*, 1235-1238.

Armstrong, R.C., Aja, T., Xiang, J.L., Gaur, S., Krebs, J.F., Hoang, K., Bai, X., Korsmeyer, J., Karanewsky, D.S., Fritz, L.C., and Tomaselli, K.J. (1996). Fas-induced activation of the cell death-related protease CPP32 is inhibited by BCL-2 and by ICE family protease inhibitors. J. Biol. Chem. *271*, 16850-16855.

Aruffo, A. and Seed, B. (1987). Molecular cloning of a CD28 cDNA by a high efficiency COS cell expression system. Proc. Natl. Acad. Sci. U.S.A. *84*, 8573-8577.

Ashany, D., Song, X., Lacy, E., Nikoliczugic, J., Friedman, S.M., and Elkon, K.B. (1995). Th1 CD4+ lymphocytes delete activated macrophages through the Fas/Apo-1 pathway. Arthritis Rheum. *38*, 685.

Ashton-Rickardt, P.G., Bandeira, A., Delaney, J.R., Vankaer, L., Pircher, H.P., Zinkernagel, R.M., and Tonegawa, S. (1994). Evidence for a differential avidity model of T-cell selection in the thymus. Cell *76*, 651-663.

Attardi, G. and Schatz, G. (1988). Biogenesis of mitochondria. Ann. Rev. Cell Biol. *4*, 289-333.

August, A., Gibson, S., Kawakami, Y., Mills, G.B., and Dupont, B. (1994). CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human leukaemic T cell line. Proc. Natl. Acad. Sci. U.S.A. *91*, 9347-9351.

Aversa, G., Chang, C.C.J., Carballido, J.M., Cocks, B.G., and de Vries, J.E. (1997). Engagement of the signaling lymphocytic activation molecule (SLAM) on activated T cells results in IL-2-independent, cyclosporin A- sensitive T cell proliferation and IFNγ production. J. Immunol. *158*, 4036-4044.

Azuma, M., Ito, D., Yagita, H., Okumura, K., Phillips, J., Lanier, L., and Somoza, C. (1993). B70 antigen is a second ligand for CTLA-4 and CD28. Nature *366*, 76-79.

Babbitt, B.P., Allen, P.M., Matsueda, G., Haber, E., and Unanue, R.R. (1985). Binding of immunogenic peptides to Ia histocompatibility molecules. Nature *317*, 359-360.

Badley, A.D., Dockrell, D., Simpson, M., Schut, R., Lynch, D.H., Leibson, P., and Paya, C.V. (1997). Macrophage-dependent apoptosis of CD4+ T lymphocytes from HIV- infected individuals is mediated by FasL and tumor necrosis factor. J. Exp. Med. *185*, 55-64.

Baichwal, V.R. and Baeuerle, P.A. (1997). Apoptosis: activate NF-κB or die? Curr. Biol. *7*, R94-R96.

Bass, H., Mosmann, T., and Strober, S. (1989). Evidence for mouse Th1-like and Th2-like helper T-cells *in vivo*- selective reduction of Th1-like cells after total lymphoid irradiation. J. Exp. Med. *170*, 1495-1511.

Benoist, C. and Mathis, D. (1989). Positive selection of the T cell repertoire - where and when does it occur? Cell *58*, 1027-1033.

Berridge, M.J. and Irvine, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature *312*, 315-321.

Bissonnette, R.P., Echeverri, F., Mahboubi, A., and Green, D.R. (1992). Apoptotic cell-death induced by *c-myc* is inhibited by *bcl-2*. Nature *359*, 552-554.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., and Wiley, D.C. (1987). Structure of the human class-I histocompatibility antigen, HLA-A2. Nature *329*, 506-512.

Blazar, B.R., Taylor, P.A., Linsley, P.S., and Vallera, D.A. (1994). *In vivo* blockade of CD28/CTLA-4 - B7/BB1 interaction with CTLA-4-Ig reduces lethal murine graft-versus-host disease across the major histocompatibility complex barrier in mice. Blood *83*, 3815-3825.

Bluestone, J.A. (1997). Is CTLA-4 a master switch for peripheral tolerance. J. Immunol. *158*, 1989-1993.

Boehme, S.A. and Lenardo, M.J. (1993). Propriocidal apoptosis of mature T lymphocytes occurs at S phase of the cell cycle. Eur. J. Immunol. *23*, 1552-1560.

Boehme, S.A., Zheng, L.X., and Lenardo, M.J. (1995). Analysis of the CD4 coreceptor and activation-induced costimulatory molecules in antigen-mediated mature T-lymphocyte death. J. Immunol. *155*, 1703-1712.

Boise, L.H., Gonzalez-Garcia, M., Postema, C., Ding, L., Lindsten, T., Turka, L.A, Mao, X., Nunez, G., and Thompson, C. (1993). BCL-X, a BCL-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*, 597-608.

Boise, L.H., Minn, A.J., Noel, P.J., June, C.H., Accavitti, M.A., Lindsten, T., and Thompson, C.B. (1995). CD28 costimulation can promote T cell survival by enhancing expression of BCL-X_L. Immunity *3*, 87-98.

Boise, L.H. and Thompson, C.B. (1997). BCL-X_L can inhibit apoptosis in cells that have undergone Fas-induced protease activation. Proc. Natl. Acad. Sci. U.S.A. *94*, 3759-3764.

Boldin, M.P., Mett, I.L., Varfolomeev, E.E., Chumakov, I., Shemer-Avni, Y., Camonis, J.H., and Wallach, D. (1995). Self-association of the 'death domains' of the p55 tumor necrosis factor (TNF) receptor and Fas/Apo1 prompts signaling for TNF and Fas/Apo1 effects. J. Biol. Chem. *270*, 387-391.

Boldin, M.P., Goncharov, T.M., Goltsev, Y.V., and Wallach, D. (1996). Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell *85*, 803-815.

Boshell, M., McLeod, J., Walker, L., Hall, N., Patel, Y., and Sansom, D. (1996). Effect of antigen presentation on superantigen induced apoptosis mediated by Fas / Fas ligand interactions in human T cells. Immunol. *87*, 586-592.

Boucher, L-M., Wiegmann, K., Futterer, A., Pfeffer, K., Machleidt, T., Schutze, S., Mak, T., and Kronke, M. (1995). CD28 Signals through acidic sphingomyelinase. J. Exp. Med. *181*, 2059-2068.

Boyum, A. (1964). Separation of white blood cells. Nature *204*, 793-796.

Brocker, T. and Karjalainen, K. (1995). Signals through TCRζ chain alone are sufficient to prime resting T lymphocytes. J. Exp. Med. *181*, 1653-1659.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1993). Three-dimensional structure of the human class-II histocompatibility antigen HLA-DR1. Nature *364*, 33-39.

Brunet, J.F, Denziot, F., Luciani, M.F, Roux-Dosseto, M., Suzan, M., Mattei, M.G, and Golstein, P. (1987). A new member of the Immunoglobulin superfamily-CTLA-4. Nature *328*, 267-270.

Brunner, T., Mogil, R.J., LaFace, D., Yoo, N.J., Mahboubi, A., Echeverri, F., Martin, S.J., Force, W.R., Lynch, D.H., Ware, C.F., and Green, D.R. (1995). Cell autonomous Fas (CD95)/ Fas-Ligand interaction mediates activation-induced apoptosis in T cell hybridomas. Nature *373*, 441-444.

Burgering, B.M.T. and Coffer, P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599-602.

Buxser, S., Bonventre, P.F., and Archer, D.L. (1981). Specific receptor-binding of Staphylococcal enterotoxins by murine splenic lymphocytes. Infect. Immun. *33*, 827-833.

Cai, Y., Cefai, D., Schneider, H., Raab, M., Nabavi, N., and Rudd, C.E. (1995). Selective CD28 pYMNM mutations implicate phosphatidyl inositol 3-kinase in CD86-CD28 mediated costimulations. Immunity *3*, 417-426.

Cascino, I., Fiucci, G., Papoff, G., and Ruberti, G. (1995). Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. J. Immunol. *154*, 2706-2713.

Cascino, I., Papoff, G., Demaria, R., Testi, R., and Ruberti, G. (1996). Fas/Apo-1 (CD95) receptor lacking the intracytoplasmic signaling domain protects tumor-cells from Fas-mediated apoptosis. J. Immunol. *156*, 13-17.

Castedo, M., Hirsch, T., Susin, S.A., Zamzami, N., Marchetti, P., Macho, A., and Kroemer, G. (1996). Sequential acquisition of mitochondrial and plasma-membrane alterations during early lymphocyte apoptosis. J. Immunol. *157*, 512-521.

Castro, J.E., Listman, J.A., Jacobson, B.A., Wang, Y.S., Lopez, P.A., Ju, S.T., Finn, P.W., and Perkins, D.L. (1996). Fas modulation of apoptosis during negative selection of thymocytes. Immunity *5*, 617-627.

Chan, A.C., van Oers, N.S.C., Tran, A., Turka, L., Law, C., Ryan, J.C., Clark, E.A., and Weiss, A. (1994). Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signalling. J. Immunol. *152*, 4758-4766.

Chan, A.C., Dalton, M., Johnson, R., Kong, G., Wang, T., Thoma, R., and Kurosaki, T. (1995). Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. EMBO J. *14*, 2499-2508.

Cheng, J., Zhou, T., Liu, C., Shapiro, J.P., Brauer, M., Kiefer, M.C., Barr, P.J., and Mountz, J.D (1994). Protection from Fas-Mediated apoptosis by a soluble form of the Fas molecule. Science *263*, 1759-1762.

Chein, Y., Gascoine, N.R., Kavaler, J., Lee, N.E., and Davis, M.M (1984). Somatic recombination in a murine T-cell receptor. Nature *309*, 322-326.

Chinnaiyan, A.M., Orourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505-512.

Chinnaiyan, A.M., Orourke, K., Yu, G.L., Lyons, R.H., Garg, M., Duan, D.R., Xing, L., Gentz, R., Ni, J., and Dixit, V.M. (1996). Signal-transduction by DR3, a

death domain-containing receptor- related to TNFR-1 and CD95. Science *274*, 990-992.

Chiu, V.K., Walsh, C.M., Liu, C.C., Reed, J.C., and Clark, W.R. (1995). BCL-2 blocks degranulation but not Fas-based cell-mediated cytotoxicity. J. Immunol. *154*, 2023-2032.

Chomczynski, P. and Sacchi, N. (1987). Single step method of RNA extraction by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156-159.

Choremipapadopoulou, H., Viglis, V., Gargalianos, P., Kordossis, R., Iniotakitheodoraki, A., and Kosmidis, J. (1994). Down-regulation of CD28 surface-antigen on CD4+ and CD8+ T- lymphocytes during HIV-1 infection. J. Acq. Imm. Def. Synd. *7*, 245-253.

Chu, J.L., Drappa, J., Parnassa, A., and Elkon, K.B. (1993). The defect in Fas mRNA expression in the MRL/*lpr* mouse is associated with insertion of the retrotransposon, *ETn*. J. Exp. Med. *178*, 723-730.

Chu, J.L., Ramos, P., Rosendorff, A., Nikoliczugic, J., Lacy, E., Matsuzawa, A., and Elkon, K.B. (1995). Massive up-regulation of the Fas ligand in *lpr* and *gld* mice - implications for Fas regulation and the graft-versus-host disease- like wasting syndrome. J. Exp. Med. *181*, 393-398.

Chuang, E., Alegre, M.L., Duckett, C.S., Noel, P.J., VanderHeiden, M.G., and Thompson, C.B. (1997). Interaction of CTLA-4 with the clathrin-associated protein AP50 results in ligand-independent endocytosis that limits cell surface expression. J. Immunol. *159*, 144-151.

Cifone, M.G., De Maria, R., Roncaioli, P., Rippo, M.R., Azuma, M., Lanier, L., Santoni, A., and Testi, R. (1993). Apoptotic signalling through CD95 (Fas/Apo-1) activates acidic sphingomyelinase. J. Exp. Med. *177*, 1547-1552.

Cleveland, J.L. and Ihle, J.N. (1995). Contenders in FasL/TNF death signaling. Cell *81*, 479-482.

Couez, D., Pages, F., Ragueneau, M., Nunes, J., Klasen, S., Mawas, C., Truneh, A., and Olive, D. (1994). Functional expression of human CD28 in murine T-cell hybridomas. Mol. Immunol. *31*, 47-57.

Damle, N., Leytze, G., Klussman, K., and Ledbetter, J. (1993). Activation with superantigen induces programmed death in antigen-primed CD4+ classII+ major histocompatability complex T lymphocytes via a CD11a/CD18-dependent mechanism. Eur. J. Immunol. *23*, 1513-1522.

Damle, N.K., Klussman, K., Linsley, P.S., and Aruffo, A. (1992). Differential costimulatory effects of adhesion molecules, B7, ICAM-1, LFA-3 and VCAM-1 on resting and antigen primed CD4+ T lymphocytes. J. Immunol. *148*, 1985-1992.

Dariavach, P., Matthei, M.-G., Golstein, P., and Lefranc, M.-P. (1988). Human Ig Superfamily CTLA4 gene: chromosomal localisation and identification of protein sequence between murine and human CTLA4 cytoplasmic domains. Eur. J. Immunol. *18*, 1901-1900.

Darzynkiewicz, Z., Bruno, S., DelBino, G., Gorczyca, W., Hotz, M.A., Lassota, P., and Traganos, F. (1992). Features of apoptotic cells measured by flow cytometry. Cytometry *13*, 795-808.

Davignon, J.L., Budd, R.C., Ceredig, R., Piguet, P.F., MacDonald, H.R., Cerottini, J.C., Vassalli, P., and Izui, S. (1985). Functional-analysis of T-cell subsets from mice bearing the *lpr* gene. J. Immunol. *135*, 2423-2428.

Davis, P.S., Hill, C.H., Keech, E., Lawton, G., Nixon, J.S., Sedgwick, A.D., Wadsworth, J., Westmacott, D., and Wilkinson, S.E. (1989). Potent selective inhibitors of protein kinase C. FEBS Lett. *259*, 61-63.

Debatin, K.M., Suss, D., and Krammer, P.H. (1994). Differential expression of APO-1 on human thymocytes - implications for negative selection. Eur. J. Immunol. *24*, 753-758.

DeBenedette, M.A., Shahinian, A., Mak, T.W., and Watts, T.H. (1997). Costimulation of CD28(-) T lymphocytes by 4-1BB ligand. J. Immunol. *158*, 551-559.

Dhein, J., Daniel, P.T., Trauth, B.C., Oehm, A., Moller, P., and Krammer, P.H. (1992). Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 surface antigens. J. Immunol. *149*, 3166-3173.

Dhein, J., Walczak, H., Baumler, C., Debatin, K-M., and Krammer, P.H. (1995). Autocrine T cell suicide mediated by APO-1/(Fas/CD95). Nature *373*, 438-441.

Dobrowsky, R.T. and Hannun, Y.A. (1992). Ceramide stimulates a cytosolic protein phosphatase. J. Biol. Chem. *267*, 5048-5051.

Dohlsten, M., Bjorklund, M., Sunstedt, A., Sansom, D., and Kalland, T. (1993). Immunopharmacology of the superantigen Staphylococcal enterotoxin A in T cell receptor Vβ3 transgenic mice. Immunol. *79*, 520-527.

Dong, L., Pittenger, G.L., and Vinik, A.I. (1995). Role of Fas in neuronal cell apoptosis induced by sera of IDDM patients with neuropathy. Mol. Biol. Cell *6*, 2060.

Downward, J. (1995). A target for PI3-kinase. Nature *376*, 553-554.

Doyle, C. and Strominger, J.L. (1987). Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature *330*, 256-258.

Duan, H.J., Chinnaiyan, A.M., Hudson, P.L., Wing, J.P., He, W.W., and Dixit, V.M. (1996). ICE/lap3, a novel mammalian homolog of the *Caenorhabditis elegans* cell-death protein ced-3 is activated during Fas and tumor necrosis factor-induced apoptosis. J. Biol. Chem. *271*, 1621-1625.

Dubey, C., Croft, M., and Swain, S.L. (1996). Naive and effector CD4 T cells differ in their requirements for T cell receptor *versus* costimulatory signals. J. Immunol. *157*, 3280-3289.

Edmead, C.E., Patel, Y.I., Wilson, A., Boulougouris, G., Hall, N.D., Ward, S.G., and Sansom, D.M. (1996). Induction of NFκB and AP-1 by CD28 signalling involves both PI3-kinase and acidic sphingomyelinase signals. J. Immunol. *57*, 3290-3297.

Eischen, C.M., Dick, C.J., and Leibson, P.J. (1994). Tyrosine kinase activation provides an early and requisite signal for Fas-induced apoptosis. J. Immunol. *153*, 1947-1954.

Eischen, C.M., Williams, B.L., Zhang, W.G., Samelson, L.E., Lynch, D.H., Abraham, R.T., and Leibson, P.J. (1997). Zap-70 tyrosine kinase is required for

the up-regulation of Fas ligand in activation-induced T cell apoptosis. J. Immunol. *159*, 1135-1139.

Ellis, J., Chain, B.M., Davies, D. H., Ibrahim, M. A. A., Katz, D. R. , Kaye, P. M., and Lightstone, E. (1991). Antigen presentation by dendritic cells provides optimal stimulation for the production of interleukin (IL)-2, IL-4 and interferon-γ by allogeneic T cells. Eur. J. Immunol. *21*, 2803-2809.

Else, K.J., Entwistle, G.M., and Grencis, R.K. (1993). Correlations between worm burden and markers of Th1 and Th2 cell subset induction in an inbred strain of mouse infected with *Trichuris muris*. Parasite Immunol. *15*, 595-600.

Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbanda, S., Robertson, M., Ghayur, T., Wong, W.W., Kamen, R., Weichselbaum, R., and Kufe, D. (1995). Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. EMBO J. *14*, 6148-6156.

Enari, M., Talanian, R.V., Wong, W.W., and Nagata, S. (1996). Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature *380*, 723-726.

Estaquier, J., Idziorek, T., Debels, F., Barresinoussi, F., Hurtrel, B., Aubertin, A.M., Venet, A., Mehtali, M., Muchmore, E., Michel, P., Mouton, Y., Girard, M., and Ameisen, J.C. (1994). Programmed cell-death and AIDS - significance of T-cell apoptosis in pathogenic and nonpathogenic primate lentiviral infections. Proc. Natl. Acad. Sci. U.S.A. *91*, 9431-9435.

Estaquier, J., Idziorek, T., Zou, W.P., Emilie, D., Farber, C.M., Bourez, J.M., and Ameisen, J.C. (1995). T-helper type 1/T helper type-2 cytokines and T-cell death - preventive effect of interleukin-12 on activation-induced and CD95 (Fas/Apo-1)-mediated apoptosis of CD4(+) T-cells from human immunodeficiency virus-infected persons. J. Exp. Med. *182*, 1759-1767.

Ettinger, R., Panka, D.J., Wang, J.K., Stanger, B.Z., Ju, S., and Marshak-Rothstein, A. (1995). Fas-Ligand mediated cytotoxicity is directly responsible for apoptosis of normal CD4+ T cells responding to a bacterial superantigen. J. Immunol. *154*, 1041-1047.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z., and Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell *69*, 119-128.

Fanidi, A., Harrington, E.A., and Evan, G.I. (1992). Cooperative interaction between *c-myc* and *bcl-2* proto-oncogenes. Nature *359*, 554-556.

Fernandez-Ruiz, E., Somoza, C., Sanchez-Madrid, F., and Lanier, L. (1995). CD28/CTLA-4 ligands - the genes encoding CD86 (B70 / B7.2) map to the same region as CD80 (B7 / B7.1) gene in human chromosome 3Q13-Q23. Eur. J. Immunol. *25*, 1453-1456.

Finck, B.K., Linsley, P.S., and Wofsy, D. (1994). Treatment of murine lupus with CTLA4-Ig. Science *265*, 1225-1227.

Finkel, T.H., Cambier, J.C., Kubo, R.T., Born, W.K., Marrack, P., and Kappler, J.W. (1989). The thymus has two functionally distinct populations of immature αβ+ T-cells - one population is deleted by ligation of αβ - TCR. Cell *58*, 1047-1054.

Fiorentino, D.F., Bond, M.W., and Mosmann, T.R. (1989). Two types of mouse T-helper cell: Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. *170*, 2081-2095.

Fisher, G.H., Rosenberg, F.J., Straus, S.E., Dale, J.K., Middelton, L.A., Lin, A.Y., Strober, W., Lenardo, M.J., and Puck, J.M. (1995). Dominant interfering Fas gene-mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell *81*, 935-946.

Fleischer, J., Soeth, E., Reiling, N., Grage-Greibenow, E., Flad, H., and Ernst, M. (1996). Differential expression and function of CD80 (B7-1) and CD86(B7-2) on human peripheral blood monocytes. Immunol. *89*, 592-598.

Foote, L.C., Schneider, T.J., Fischer, G.M., Wang, J.K.M., Rasmussen, B., Campbell, K.A., Lynch, D.H., Ju, S.T., Marshak-Rothstein, A., and Rothstein, T.L. (1996). Intracellular signaling for inducible antigen receptor-mediated Fas resistance in B-cells. J. Immunol. *157*, 1878-1885.

Fournel, S., Genestier, L., Ronbinet, E., Flacher, M., and Revillard, J.-P. (1996). Human T cells require IL-2 but not G1/S transition to acquire susceptibility to Fas-mediated apoptosis. J. Immunol. *157*, 4309-4315.

Franke, T.F., Yang, S., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R., and Tsichlis, P.N. (1995). The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidlyinositol 3-kinase. Cell *81*, 727-736.

Fraser, J.D, Irving, B.A., Crabtree, G.R, and Weiss, A. (1991). Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. Science *251*, 313-316.

Fraser, J.D. and Weiss, A. (1992). Regulation of T cell lymphokine gene transcription by the accessory molecule CD28. Mol. Cell. Biol. *12*, 4357-4363.

Freeman, G.J, Freedman, A.S., Segil, J.M., Lee, G., Whitman, J.F, and Nadler, L.M (1989). B7 a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. J. Immunol. *143*, 2714-2710.

Freeman, G.J., Gribben, J.G., Boussiotis, V.A., Ng, J.W., Restivo, V.A., Lombard, L.A., Gray, G.S, and Nadler, L.M (1993). Cloning of B7-2: A CTLA-4 counter-receptor that costimulates human T cell proliferation. Science *262*, 909-912.

Freeman, G.J., Boussiotis, V.A., Anumanthan, A., Bernstein, G.M., Ke, X., Rennert, P., Gray, G.S., Gribben, J.G., and Nadler, L.M. (1995). B7-1and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. Immunity *2*, 523-532.

French, L.E., Wilson, A., Hahne, M., Viard, I., Tschopp, J., and MacDonald, H.R. (1997). Fas ligand expression is restricted to non-lymphoid thymic components *in situ*. J. Immunol. *159*, 2196-2202.

Gajewski, T.F. and Thompson, C.B. (1996). Apoptosis meets signal transduction: elimination of a BAD influence. Cell *87*, 589-592.

Gamen, S., Marzo, I., Anel, A., Pineiro, A., and Naval, J. (1996). CPP32 inhibition prevents Fas-induced ceramide generation and apoptosis in human cells. FEBS Lett. *390*, 233-237.

Genot, E., Parker, P.J., and Cantrell, D. (1995). Analysis of the role of PKC α, ε and ζ in T cell activation. J. Biol. Chem. *270*, 9833-9830.

Germain, R. and Hendrix, L. (1991). MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. Nature *353*, 134-139.

Gillette-Ferguson, I. and Sidman, C.L. (1994). A specific intercellular pathway of apoptotic cell death is defective in the mature peripheral T cells of *lpr* and *gld* mice. Eur. J. Immunol. *24*, 1181-1185.

Gimmi, C.D, Freeman, G.J, Gribben, J.G., Sugita, K., Freedman, A.S., Morimoto, C., and Nadler, L.M. (1991). B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. Proc. Natl. Acad. Sci. U.S.A. *88*, 6575-6579.

Gombert, W., Borthwick, N.J., Wallace, D.L., Hyde, H., Bofill, M., Pilling, D., Beverley, P.C.L., Janossy, G., Salmon, M., and Akbar, A.N. (1996). Fibroblasts prevent apoptosis of IL-2-deprived T cells without inducing proliferation - a selective effect on BCL-X_L expression. Immunol. *89*, 397-404.

Gonzalez-Garcia, A., Rborlado, L., Leonardo, E., Merida, I., Martinez, A. C., and Carrera, A.C. (1997). *Lck* is necessary and sufficient for Fas-ligand expression and apoptotic cell death in mature cycling T cells. J. Immunol. *158*, 4104-4112.

Green, D.R. and Ware, C.F. (1997). Fas-ligand: privilege and peril. Proc. Natl. Acad. Sci. U.S.A. *94*, 5986-5990.

Greene, J.L., Leytze, G.M., Emswiler, J., Peach, R., Bajorath, J., Cosand, W., and Linsley, P.S. (1996). Covalent dimerisation of CD28/CTLA-4 and oligomerisation of CD80/CD86 regulate costimulatory interactions. J. Biol. Chem. *271*, 26762-26771.

Greenfield, E.A., Howard, E., Paradis, T., Nguyen, K., Benazzo, F., McLean, P., Hollsburg, P., Davis, G., Hafler, D.A., Sharpe, A.H., Freeman, G.J., and Kuchroo, V.K. (1997). B7.2 expressed by T cells does not induce CD28-mediated costimulatory activity but retains CTLA-4 binding- implications for induction of anti-tumour immunity. J. Immunol. *158*, 2025-2034.

Gribben, J.G., Freeman, G.J., Boussiotis, V.A., Rennert, P., Jellis, C.L., Greenfield, E., Barber, M., Restivo, V.A., Ke, X., Gray, G.S., and Nadler, L.M. (1995). CTLA-4 mediates antigen-specific apoptosis of human T cells. Proc. Natl. Acad. Sci. U.S.A. *92*, 811-815.

Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.R., and Ferguson, T.A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. Science *270*, 1189-1192.

Grillot, D.A.M., Merino, R., and Nunez, G. (1995). BCL-X_L displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. J. Exp. Med. *182*, 1973-1983.

Groux, H., Torpier, G., Monte, D., Mouton, Y., Capron, A., and Ameisen, J-C. (1992). Activation-induced death by apoptosis in CD4+ T cells from Human Immunodeficiency Virus-infected individuals. J. Exp. Med. *175*, 331-340.

Groux, H., Monte, D., Plouvier, B., Capron, A., and Ameisen, J-C. (1993). CD3-mediated apoptosis of human medullary thymocytes and activated peripheral T cells: respective roles of interleukin-1, interleukin-2, interferonγ and accessory cells. Eur. J. Immunol. *23*, 1623-1629.

Gulbins, E., Bissonnette, R., Mahboubi, A., Martin, S., Nishioka, W., Brunner, T., Baier, G., Baierbitterlich, G., Byrd, C., Lang, F., Kolesnick, R., Altman, A., and Green, D. (1995). Fas-induced apoptosis is mediated via a ceramide-initiated *ras* signaling pathway. Immunity *2*, 341-351.

Hanabuchi, S., Koyanagi, M., Kawasaki, A., Shinohara, N., Matsuzawa, A., Nishimura, Y., Kobayashi, Y., Yonehara, S., Yagita, H., and Okumura, K. (1994). Fas and its ligand in a general mechanism of T-cell-mediated cytotoxicity. Proc. Natl. Acad. Sci. U.S.A. *91*, 4930-4934.

Hannun, Y.A. (1994). The sphingomyelin cycle and second messenger function of ceramide. J. Biol. Chem. *269*, 3125-3128.

Hannun, Y.A. and Obeid, L.M. (1995). Ceramide - an intracellular signal for apoptosis. TIBS *20*, 73-77.

Hargreaves, R.G., Borthwick, N.J., Montani, M.S.G., Piccolella, E., Carmichael, P., Lechler, R.I., Akbar, A.N., and Lombardi, G. (1997). Dissociation of T cell

anergy from apoptosis by blockade of Fas/Apo-1 (CD95) signalling. J. Immunol. *158*, 3099-3107.

Hengartner, M.O., Ellis, R.E., and Horvitz, H.R. (1992). *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell-death. Nature *356*, 494-499.

Hengartner, M.O. (1994). Programmed cell death - a rich harvest. Curr. Biol. *4*, 950-952.

Hengartner, M.O. and Horvitz, H.R. (1994). *C. elegans* cell-survival gene *ced-9* encodes a functional homologue of the mammalian proto-oncogene *bcl-2*. Cell *76*, 665-676.

Hengartner, M.O. (1996). Programmed cell-death in invertebrates. Curr. Opin. Gen. & Devel. *6*, 34-38.

Henkart, P.A. (1996). ICE family proteases - mediators of all apoptotic cell-death. Immunity *4*, 195-201.

Herman, A., Kappler, J., Marrack, P., and Pullen, A. (1991). Superantigens: mechanisms of T cell stimulation and role in immune responses. Ann. Rev. Immunol. *9*, 745-773.

Herron, L.R., Eisenberg, R.A., Roper, E., Kakkanaiah, V.N., Cohen, P.L., and Kotzin, B.L. (1993). Selection of the T-cell receptor repertoire in *lpr* mice. J. Immunol. *151*, 3450-3459.

Higuchi, M., Singh, S., Jaffrezou, J.P., and Aggarwal, B.B. (1996). Acidic sphingomyelinase-generated ceramide is needed but not sufficient for TNF-induced apoptosis and nuclear factor-k-B activation. J. Immunol. *157*, 297-304.

Hiramatsu, N., Hayashi, N., Katayama, K., Mochizuki, K., Kawanishi, Y., Kasahara, A., Fusamoto, H., and Kamada, T. (1994). Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic Hepatitis C. Hepatol *19*, 1354-1359.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., and Korsmeyer, S.J. (1993). BCL-2 functions in an antioxidant pathway to prevent apoptosis. Cell *75*, 241-251.

Hockenbery, M.O., Nunez, Z., Milliman, C., Schreiber, R.D., and Korsmeyer, S.J. (1990). BCL-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature *348*, 334.

Houssaint, E. and Flajnik, M. (1990). The role of thymic epithelium in acquisition of tolerance. Immunol. Today *11*, 357-360.

Howard, F.D., Moingeon, P., Moebius, U., McConkey, D.J., Yandava, B., Gennert, T.E., and Reinhertz, E.L. (1992). The CD3-ζ cytoplasmic domain mediates CD2-induced T cell activation. J. Exp. Med. *176*, 139-130.

Huang, L. and Crispe, I.N. (1993). Superantigen-driven peripheral deletion of T-cells - apoptosis occurs in cells that have lost the αβ T-cell receptor. J. Immunol. *151*, 1844-1851.

Hughes, D.P.M. and Crispe, I.N. (1995). A naturally-occurring soluble isoform of murine Fas generated by alternative splicing. J. Exp. Med. *182*, 1395-1401.

Hyde, H., Borthwick, N.J., Janossy, G., Salmon, M., and Akbar, A.N. (1997). Upregulation of intracellular glutathione by fibroblast-derived factor(s): enhanced survival of activated T cells in the presence of low BCL-2. Blood *89*, 2453-2460.

Ibrahim, S., Jakobs, F., Linsley, P.S., Sanfilippo, F., and Baldwin, W.M. (1997). CTLA-4-Ig inhibits alloantibody responses to transfusions and transplants. Trans. Proc. *29*, 1025.

Inohara, N., Koseki, T., Hu, Y.M., Chen, S., and Nunez, G. (1997). CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. Proc. Natl. Acad. Sci. U.S.A. *94*, 10717-10722.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, B., Steiner, V., Bodmer, J.L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L.E., and Tschopp, J. (1997). Inhibition of death receptor signals by cellular FLIP. Nature *388*, 190-195.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S-I., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for the cell surface antigen Fas can mediate apoptosis. Cell *66*, 233-243.

Itoh, N., Imagawa, A., Hanafusa, T., Waguri, M., Yamamoto, K., Iwahashi, H., Moriwaki, M., Nakajima, H., Miyagawa, J., Namba, M., Makino, S., Nagata, S., Kono, N., and Matsuzawa, Y. (1997). Requirement of Fas for the development of autoimmune diabetes in nonobese diabetic mice. J. Exp. Med. *186*, 613-618.

Janeway, C.A.JR., Yagi, J., Conrad, P., Katz, M., Vroegop, S., and Buxser, S. (1989). T cell responses to Mls and to bacterial proteins that mimic its behaviour. Immunol. Rev. *107*, 61-88.

Jans, D.A., Jans, P., Briggs, L.J., Sutton, V., and Trapani, J.A. (1996). Nuclear transport of granzyme-B (fragmentin-2) - dependence on perforin *in-vivo* and cytosolic factors *in-vitro*. J. Biol. Chem. *271*, 30781-30789.

Janssen, O., Wesselborg, S., Hecklostreicher, B., Pechhold, K., Bender, A., Schondelmaier, S., Moldenhauer, G., and Kabelitz, D. (1991). T-cell receptor CD3-signaling induces death by apoptosis in human T- cell receptor γδ+ T-cells. J. Immunol. *146*, 35-39.

Janssen, O., Lengljanssen, B., Oberg, H.H., Robertson, M.J., and Kabelitz, D. (1996). Induction of cell-death via Fas (CD95, Apo-1) may be associated with but is not dependent on Fas-induced tyrosine phosphorylation. Immunol. Lett. *49*, 63-69.

Jayadev, S., Liu, B., Bielawska, A.E., Lee, J.Y., Nazaire, F., Pushkareva, M.Y., Obeid, L.M., and Hannun, Y.A. (1995). Role for ceramide in cell-cycle arrest. J. Biol. Chem. *270*, 2047-2052.

Jenkins, M.K., Taylor, P.S., Norton, S.D., and Urdahl, K.B. (1991). CD28 delivers a costimulatory signal involved in antigen specific IL-2 production by human T cells. J. Immunol. *147*, 2461-2466.

Jodo, S., Kobayashi, S., Kayagaki, N., Ogura, N., Feng, Y., Amasaki, Y., Fujisaku, A., Azuma, M., Yagita, H., Okumura, K., and Koike, T. (1997). Serum levels of soluble Fas/Apo-1 (CD95) and its molecular structure in patients with systemic lupus erythematosus (SLE) and other autoimmune diseases. Clin. Exp. Immunol. *107*, 89-95.

Jones, L.A., Chin, L.T., Merriam, G.R., Nelson, L.M., and Kruisbeck, A.M. (1990). Failure of clonal deletion in neonatally thymectomised mice: tolerance is preserved through clonal anergy. J. Exp. Med. *172*, 1277-1285.

Ju, S., Panka, D.J., Cui, H., Ettinger, R., El-Khatib, ., Sherr, D.H., Stanger, B.Z., and Marshak-Rothstein, A. (1995). Fas (CD95) / FasL interactions required for programmed cell death after T cell activation. Nature *373*, 444-448.

Ju, S.T., Cui, H.L., Panka, D.J., Ettinger, R., and Marshak-Rothstein, A. (1994). Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T-cells. Proc. Natl. Acad. Sci. U.S.A. *91*, 4185-4189.

June, C.H., Bluestone, J.A., Nadler, L.M, and Thompson, C.B. (1994). The B7 and CD28 receptor families. Immunol. Today *15*, 321-331.

Kanagawa, O., Vaupel, B.A., Korsmeyer, S.J., and Russell, J.H. (1995). Apoptotic death of lymphocytes in murine acquired-immunodeficiency- syndrome - involvement of Fas-Fas ligand interaction. Eur. J. Immunol. *25*, 2421-2427.

Kanner, S.B., Damle, N.K., Blake, J., Aruffo, A., and Ledbetter, J.A. (1992). CD2/LFA3 ligation induces phospholipase C-γ1 tyrosine phosphorylation and regulates CD3 signalling. J. Immunol. *148*, 2023-2020.

Kappler, J. and Marrack, P. (1987). T cell tolerance in the thymus. Cell *49*, 273-280.

Kappler, J., Kotzin, B., Herron, L., Gelfand, E.W., Bigler, R.D., Boylston, A., Carrel, S., Posnett, D.N., Choi, Y., and Marrack, P. (1989). Vβ-specific stimulation of human T cells by Staphylococcal toxins. Science *244*, 811-813.

Kappler, J.W., Roehm, N., and Marrack, P. (1987). T-cell tolerance by clonal elimination in the thymus. Cell *49*, 273-280.

Karulin, A.Y. and Lehmann, P.V. (1997). The Th1/Th2 cell may not exists *in vivo*: paradigm challenged by double-colour cytokine ELISA spot assay. J. All. Clin. Immunol. *99*, 1490.

Kasahara, Y., Wada, T., Niida, Y., Ohno, I., Yachie, A., Seki, H., Miyawaki, T., and Taniguchi, N. (1996). Human lymphoproliferative disease associated with Fas mutation. FASEB J. *10*, 374.

Kauffman-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., and Evan, G. (1997). Suppression of *c-myc*-induced apoptosis by *ras* signalling through PI(3)K and PKB. Nature *385*, 544-548.

Kaufmann, S.H., Desnoyers, S., Ottaviano, Y., Davidson, N.E., and Poirier, G.G. (1993). Specific proteolytic cleavage of poly(ADP-ribose) polymerase - an early marker of chemotherapy-induced apoptosis. Cancer Res. *53*, 3976-3985.

Kayagaki, N., Kawasaki, A., Ebata, T., Ohmoto, H., Ikeda, S., Inoue, S., Yoshino, K., Okumura, K., and Yagita, H. (1995). Metalloproteinase-mediated release of human Fas ligand. J. Exp. Med. *182*, 1777-1783.

Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239-257.

Kirberg, J., Berns, A., and Boehmer, H. von (1997). Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. J. Exp. Med. *186*, 1269-1275.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H., and Peter, M.E. (1995). Cytotoxicity-dependent Apo-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. *14*, 5579-5588.

Kishimoto, H., Kubo, R.T., Yorifuji, H., Nakayama, T., Asano, Y., and Tada, T. (1995). Physical dissociation of the TCR-CD3 complex accompanies receptor ligation. J. Exp. Med. *182*, 1997-2006.

Kitson, J., Raven, T., Jiang, Y.P., Goeddel, D.V., Giles, K.M., Pun, K.T., Grinham, C.J., Brown, R., and Farrow, S.N. (1996). A death-domain-containing receptor that mediates apoptosis. Nature *384*, 372-375.

Klas, C., Debatin, K-M., Jonker, R.R., and Krammer, P.H. (1993). Activation interferes with the APO-1 pathway in mature human T cells. Int. Immunol. *5*, 625-630.

Klausner, R.D. and Samelson, L.E. (1991). T-cell antigen receptor activation pathways - the tyrosine kinase connection. Cell *64*, 875-878.

Kleijmeer, M.J., Kelly, A., Geuze, H.J., Slot, J.W., Townsend, A., and Trowsdale, J. (1992). Location of MHC-encoded transporters in the endoplasmic-reticulum and cis-golgi. Nature *357*, 342-344.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., and Newmeyer, D.D. (1997). The release of cytochrome c from mitochondria: a primary site for BCL-2 regulation of apoptosis. Science *275*, 1132-1136.

Kolesnick, R. and Golde, D.W. (1994). The sphingomyelin pathway in tumour necrosis factor and interleukin-1 signalling. Cell *77*, 325-328.

Kovacs, B., Szentendrei, T., Bednarek, J.M., Pierson, M.C., Mountz, J.D., Vogelgesang, S.A., and Tsokos, G.C. (1997). Persistent expression of a soluble form of Fas/Apo1 in continuously activated T cells from a patient with SLE. Clin. Exp. Rheumatol. *15*, 19-23.

Kraft, A.S. and Anderson, W.B. (1983). Phorbol esters increase the amount of Ca-2+, phospholipid-dependent protein-kinase associated with plasma-membrane. Nature *301*, 621-623.

Krummel, M.F. and Allison, J.P. (1995). CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J. Exp. Med. *182*, 459-465.

Krummel, M.F. and Allison, J.P. (1996). CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. J. Exp. Med. *183*, 2533-2540.

Kuchroo, V.K., Das, M.P., Brown, J.A., Ranger, A.M., Zamvill, S.S., Sobel, R.A., Weiner, H.L., Nabavi, N., and Glimcher, L.H. (1995). B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell *80*, 707-718.

Kulkarni, G.V. and Mcculloch, C.A.G. (1994). Serum deprivation induces apoptotic cell-death in a subset of BALB/c 3T3 fibroblasts. J. Cell Sci. *107*, 1169-1179.

Kumar, S. and Lavin, M.F. (1996). The ICE family of cysteine proteases as effectors of cell-death. Cell Death & Diff. *3*, 255-267.

Lamb, C.A., Yewdell, J.W., Bennink, J.R., and Cresswell, P. (1991). Invariant chain targets HLA class-II molecules to acidic endosomes containing internalised influenza-virus. Proc. Natl. Acad. Sci. U.S.A. *88*, 5998-6002.

Lando, P., Dohlsten, M., Hedlund, G., Brodin, T., Sansom, D., and Kalland, T. (1993). Costimulation with B7 and targeted superantigens is required for MHC class II independent T cell proliferation but not cytotoxicity. Immunol. *80*, 236-241.

Lanier, L., O'Fallon, S., Somoza, C., Phillips, J.H., Linsley, P.S., Okumura, K., Ito, D., and Azuma, M. (1995). CD80(B7) and CD86(B70) provide similar costimulatory signals for T cell proliferation, cytokine production and generation of CTL. J. Immunol. *154*, 97-105.

Latinis, K.M., Carr, L.L., Peterson, E.J., Norian, L.A., Eliason, S.L., and Koretzky, G.A. (1997). Regulation of CD95 (Fas) ligand expression by TCR-mediated signaling events. J. Immunol. *158*, 4602-4611.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G., and Earnshaw, W.C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature *371*, 346-347.

Leach, D.R., Krummel, M.F., and Allison, J.P. (1996). Enhancement of anti-tumour immunity by CTLA-4 blockade. Science *271*, 1734-1736.

Lederer, J.A., Liou, J.S., Todd, M.D., Glimcher, L.H., and Lichtman, A.H. (1993). Transcriptional regulation of IL-2 and IL-4 expression in murine Th1 and Th2 clones. J. Immunol. *150*, A 5.

Lee, S.L., Wesselschmidt, R.L., Linette, G.P., Kanagawa, O., Russell, J.H., and Milbrandt, J. (1995). Unimpaired thymic and peripheral T-cell death in mice lacking the nuclear receptor NGFI-B (Nur77). Science *269*, 532-535.

Lenardo, M.J (1991). Interleukin 2 programs mouse α/β T lymphocytes for apoptosis. Nature *353*, 858-861.

Lenschow, D., Zeng, Y., Thistlethwaite, J., Monty, A., Brady, W., Gibson, N., Linsley, P., and Bluestone, J. (1992). Longterm survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. Science *257*, 789-780.

Lenschow, D.J., Ho, S.C., Sattar, H., Rhee, L., Gray, G., Nabavi, N., Herold, K.C., and Bluestone, J.A. (1995). Differential effects of anti-B7-1 and B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. J. Exp. Med. *181*, 1145-1155.

Lenschow, D.J., Herold, K.C., Rhee, L., Patel, B., Koons, A., Qin, H.Y., Fuchs, E., Singh, B., Thompson, C.B., and Bluestone, J.A. (1996). CD28/B7 regulation of Th-1 and Th-2 subsets in the development of autoimmune diabetes. Immunity *5*, 285-293.

Li, W., Whaley, C.D., Mondino, A., and Mueller, D.L. (1996). Blocked signal-transduction to the ERK and JNK protein-kinases in anergic CD4+ T-cells. Science *271*, 1272-1276.

Liao, X.C., Fournier, S., Killeen, N., Weiss, A., Allison, J.P., and Littman, D.R. (1997). *Itk* negatively regulates induction of T cell proliferation by CD28 costimulation. J. Exp. Med. *186*, 221-228.

Liles, W.C., Kiener, P.A., Ledbetter, J.A., Aruffo, A., and Klebanoff, S.J. (1996). Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes - implications for the regulation of apoptosis in neutrophils. J. Exp. Med. *184*, 429-440.

Lindenboim, L., Diamond, R., Rothenberg, E., and Stein, R. (1995). Apoptosis induced by serum deprivation of PC12 cells is not preceded by growth arrest and can occur at each phase of the cell-cycle. Cancer Res. *55*, 1242-1247.

Lindsten, T., June, C.H., Ledbetter, J.A., Stella, G., and Thomson, C.B. (1989). Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. Science *244*, 339-330.

Linsley, P., Bradshaw, J., Urnes, M., Grosmaire, L., and Ledbetter, J. (1993). CD28 engagement by B7/BB1 induces transient down-regulation of CD28 synthesis and prolonged unresponsiveness to CD28 signalling. J. Immunol. *150*, 3161-3169.

Linsley, P.S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N.K., and Ledbetter, J.A. (1991a). Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. J. Exp. Med. *173*, 721-730.

Linsley, P.S., Brady, W., Urnes, M., Grosmaire, L., Damle, N.K., and Ledbetter, J.A. (1991b). CTLA-4 is a second receptor for the B cell activation antigen B7. J. Exp. Med. *174*, 561-569.

Linsley, P.S., Wallace, P.M., Johnson, J., Gibson, M.G., Greene, J.L., Ledbetter, J.A., Singh, C., and Tepper, M.A. (1992). Immunosuppression *in vivo* by a soluble form of the CTLA-4 T cell activation molecule. Science *257*, 792-795.

Linsley, P.S., Bradshaw, J., Urnes, M., Grosmaire, L., and Ledbetter, J.A. (1993). CD28 engagement by B7/BB-1 induces transient down-regulation of CD28 synthesis and prolonged unresponsiveness to CD28 signalling. J. Immunol. *150*, 3161-3169.

Linsley, P.S. and Ledbetter, J.A. (1993). The role of the CD28 receptor during T cell responses to antigen. Ann. Rev. Immunol. *11*, 191-212.

Linsley, P.S., Bradshaw, J., Greene, J., Peach, R., Bennett, K.L., and Mittler, R.S. (1996). Intracellular trafficking of CTLA-4 and focal localisation towards sites of TCR engagement. Immunity *4*, 535-543.

Liu, X., Kim, J., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. J. Exp. Med. *86*, 147-157.

Liu, Z.G., Smith, S.W., Mclaughlin, K.A., Schwartz, L.M., and Osborne, B.A. (1994). Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene Nur77. Nature *367*, 281-284.

Liu, Z.G., Hsu, H.L., Goeddel, D.V., and Karin, M. (1996). Dissection of TNF receptor-1 effector functions - JNK activation is not linked to apoptosis while NF-κ-B activation prevents cell death. Cell *87*, 565-576.

Lo, D., Burkly, L.C, Cowing, C., Flavell, R.A., Palmiter, R.D., and Brinster, R.L. (1988). Diabetes and tolerance in transgenic mice expressing class II MHC molecules in pancreatic β cells. Cell *53*, 159-168.

Lo, D., Burkly, L.C., Flavell, R.A., Palmiter, R.D., and Brinster, R.L. (1989). Tolerance in transgenic mice expressing class II major histocompatibility complex on pancreatic cells. J. Exp. Med. *170*, 87-104.

Los, M., Vandecraen, M., Penning, L.C., Schenk, H., Westendorp, M., Baeuerle, P.A., Droge, W., Krammer, P.H., Fiers, W., and Schulzeosthoff, K. (1995). Requirement of an ICE/ced-3 protease for Fas/Apo-1-mediated apoptosis. Nature *375*, 81-83.

Lu, L., Qian, S., Hershberger, P.A., Rudert, W.A., Lynch, D.H., and Thomson, A.W.. (1997a). Fas ligand (CD95L) and B7 expression on dendritic cells provide counter-regulatory signals for T cell survival and proliferation. J. Immunol. *158*, 5676-5684.

Lu, L., Qian, S.G., Starzl, T.E., Lynch, D.H., and Thomson, A.W. (1997b). Blocking of the B7-CD28 pathway increases the capacity of FasL(+) (CD95L) dendritic cells to kill alloactivated T cells. Adv. Expt. Med. Biol. *417*, 275-282.

Lucas, M., Sanchez-Margalet, V., Sanz, A., and Solano, F. (1994). Protein kinase C activation promotes cell survival in mature lymphocytes prone to apoptosis. Biochem. Pharmacol. *47*, 667-672.

Lucas, P.J., Negishi, I., Nakayama, K., Fields, L.E., and Loh, D.Y. (1995). Naive CD28 deficient T cells can initiate but not sustain an *in vitro* antigen specific immune response. J. Immunol. *154*, 5757-5768.

Luciani, M.F. and Golstein, P. (1994). Fas-based D10S-mediated cytotoxicity requires macromolecular- synthesis for effector cell activation but not for target-cell death. Phil. Trans. Royal Soc. Lond. *345*, 303-309.

MacDonald, H.R, Bashieri, S., and Lees, R.K (1991). Clonal expansion preceeds anergy and death of $V\beta 8^+$ positive peripheral T cells responding to Staphylococcal enterotoxin B *in vivo*. Eur. J. Immunol. *21*, 1963-1966.

MacLennan, I.C.M., Gulbranson-Judge, A., Toellner, K.M., Casamayor-Palleja, M., Chan, E., Sze, D.M.Y., Luther, S.A., and Orbea, H.A. (1997). The changing preference of T and B cells for partners as T-dependent antibody responses develop. Immunol. Rev. *156*, 53-66.

Mariani, S.M., Matiba, B., Baumler, C., and Krammer, P.H. (1995). Regulation of cell-surface Apo-1/Fas (CD95) ligand expression by metalloproteases. Eur. J. Immunol. *25*, 2303-2307.

Markmann, J., Lo, D., Naji, A., Palmiter, R.D., Brinster, R.L., and Heber-Katz, E. (1988). Antigen presenting function of class II MHC expressing pancreatic β cells. Nature *336*, 476-479.

Marrack, P. and Kappler, J. (1987). The T-cell receptor. Science *238*, 1073-1079.

Martin, S.J. and Green, D.R. (1995). Protease activation during apoptosis - death by a thousand cuts? Cell *82*, 349-352.

Martin, S.J., Obrien, G.A., Nishioka, W.K., Mcgahon, A.J., Mahboubi, A., Saido, T.C., and Green, D.R. (1995a). Proteolysis of fodrin (nonerythroid spectrin) during apoptosis. J. Biol. Chem. *270*, 6425-6428.

Martin, S.J., Reutelingsperger, C.P.M., Mcgahon, A.J., Rader, J.A., Vanschie, R.C.A.A., Laface, D.M., and Green, D.R. (1995b). Early redistribution of plasma-membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus - inhibition by overexpression of *bcl-2* and *abl*. J. Exp. Med. *182*, 1545-1556.

Maryanski, J.L., Casanova, J.L., Falk, K., Gournier, H., Jaulin, C., Kourilsky, P., Lemonnier, F.A., Luthy, R., Rammensee, H.G., Rotzschke, O., Servis, C., and Lopez, J.A. (1997). The diversity of antigen-specific TCR repertoires reflects the relative complexity of epitopes recognised. Human Immunol. *54*, 117-128.

Mathias, S., Dressler, K.A., and Kolesnick, R.N. (1991). Characterisation of a ceramide-activated protein-kinase - stimulation by tumor-necrosis-factor-α. Proc. Natl. Acad. Sci. U.S.A. *88*, 10009-10013.

Matzinger, P. (1991). The JAM test: a simple assay for DNA fragmentation and cell death. J. Immunol. Meths. *145*, 185-189.

McConkey, D.J., Nicotera, P., and Orrenius, S. (1994). Signaling and chromatin fragmentation in thymocyte apoptosis. Immunol. Rev. *142*, 343-363.

Mcgahon, A.J., Nishioka, W.K., Martin, S.J., Mahboubi, A., Cotter, T.G., and Green, D.R. (1995). Regulation of the Fas apoptotic cell-death pathway by *abl*. J. Biol. Chem. *270*, 22625-22631.

McLeod, J.D., Walker, L.S.K., Ellwood, C., Patel, Y.I., Boulougouris, G., and Sansom, D.M. (1997). Activation of human T cells with superantigen and CD28 confers resistance to apoptosis by CD95. J. Immunol. In Press.

Medema, J.P., Scaffidi, C., Kischkel, F.C., Shevchenko, A., Mann, M., Krammer, P.H., and Peter, M.E. (1997). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J. *16*, 2794-2804.

Miller, S.D., Vanderlugt, C.L., Lenschow, D.J., Pope, J.G., Karandikar, N.J., Dal Canto, M.C., and Bluestone, J.A. (1995). Blockade of CD28/B7-1 interaction prevents epitope spreading and clinical relapses in murine EAE. Immunity *3*, 739-745.

Miyawaki, T., Uehara, T., Nibu, R., Tsuji, T., Yachi, A., Yonehara, S., and Taniguchi, N. (1992). Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. J. Immunol. *149*, 3753-3758.

Moreno, M.B., Memon, S.A., and Zacharchuk, C.M. (1996). Apoptosis signaling pathways in normal T cells. J. Immunol. *157*, 3845-3849.

Morris, P., Shaman, J., Attaya, J., Amaya, M., Goodman, M., Bergman, S., Monaco, C., and Mellins, E. (1994). An essential role for HLA-DM in antigen presentation by class II Major Histocompatibility Molecules. Nature *368*, 551-554.

Mosmann, T.R. and Coffman, R.L. (1989). Th1-cell and Th2-cell - different patterns of lymphokine secretion lead to different functional-properties. Ann. Rev. Immunol. *7*, 145-173.

Mueller, D.L., Seiffert, S., Fang, W., and Behrens, T.W. (1996). Differential regulation of BCL-2 and BCL-X by CD3, CD28, and the IL-2 receptor in cloned CD4+ helper T-cells - a model for the long-term survival of memory cells. J. Immunol. *156*, 1764-1771.

Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., Orourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E., and Dixit, V.M. (1996). FLICE, a novel FADD-homologous ICE/ced-3-like protease, is recruited to the CD95 (Fas/Apo-1) death-inducing signaling complex. Cell *85*, 817-827.

Nagata, S. (1994). Apoptosis regulated by a death factor and its receptor: Fas ligand and Fas. Phil. Trans. Royal Soc. Lond. *345*, 281-287.

Nakajima, H., Golstein, P., and Henkart, P.A. (1995). The target-cell nucleus is not required for cell-mediated granzyme- based or Fas-based cytotoxicity. J. Exp. Med. *181*, 1905-1909.

Neamati, N., Fernandez, A., Wright, S., Kiefer, J., and McConkey, D.J. (1995). Degradation of lamin B-1 precedes oligonucleosomal DNA fragmentation in apoptotic thymocytes and isolated thymocyte nuclei. J. Immunol. *154*, 3788-3795.

Negoescu, A., Lorimier, P., Labatmoleur, F., Drouet, C., Robert, C., Guillermet, C., Brambilla, C., and Brambilla, E. (1996). *In-situ* apoptotic cell labelling by the TUNEL method - improvement and evaluation on cell preparations. J. Hist. Cyt.*44*, 959-968.

Noble, A., Kemeny, D.M., and Staynov, D.Z. (1993). Generation of Th1- and Th2-like cells *in vitro* is regulated by IL-4 and IFNγ. J. Immunol. *150*, A 274.

Noel, P.J., Boise, L.H., Green, J.M., and Thompson, C.B. (1996a). CD28 costimulation prevents cell death during primary T cell activation. J. Immunol. *157*, 636-642.

Noel, P.J., Boise, L.H., and Thompson, C.B. (1996b). Regulation of T cell activation by CD28 and CTLA4. In Mechanisms of Lymphocyte Activation and Immune Regulation VI. Gupta and Cohen, eds. (New York: Plenum Press), pp. 209-217.

Nunez, G., Merino, R., Grillot, D., and Gonzalez-Garcia, M. (1994). BCL-2 and BCL-X - regulatory switches for lymphoid death and survival. Immunol. Today *15*, 582-588.

Oehm, A., Behrmann, I., Falk, W., Pawlita, M., Maier, G., Klas, C., Li-Weber, M., Richards, S., Dhein, J., Trauth, B.C., Ponsting, H., and Krammer, P.H. (1992). Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. J. Biol. Chem. *67*, 10709-10713.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., and Nagata, S. (1993). Lethal effect of the anti-Fas antibody in mice. Nature, Lond. *364*, 806-809.

Ogasawara, J., Suda, T., and Nagata, S. (1995). Selective apoptosis of CD4+ CD8+ thymocytes by the anti-Fas antibody. J. Exp. Med. *181*, 485-491.

Okabe, T., Takayanagi, R., Imasaki, K., Haji, M., Nawata, H., and Watanabe, T. (1995). Cloning of a NGFI-B/ Nur77 related transcription factor from an apoptotic human T cell line. J. Immunol. *154*, 3871-3879.

Okubo, M., Ishida, H., Nishimaki, T., Yagita, H., Okumura, K.O., and Kasukawa, R. (1996). Levels of soluble Fas but not soluble Fas ligand were elevated in the sera of patients with SLE. Arthritis Rheum. *39*, 103.

Okura, T., Gong, L., Kamitani, T., Wada, T., Okura, I., Wei, C.-R., Chang, H.-M., and Yeh, E.T.H. (1996). Protection against Fas/Apo-1- and tumor necrosis factor-mediated cell death by a novel protein, Sentrin. J. Immunol. *156*, 4277-4281.

Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. (1993). BCL-2 heterodimerizes *in vivo* with a conserved homologue, BAX, that accelerates programmed cell-death. Cell *74*, 609-619.

Ormerod, M.G. (1990). Flow cytometry: a practical approach (Oxford: IRL Press).

Orth, K., Chinnaiyan, A.M., Garg, M., Froelich, C.J., and Dixit, V.M. (1996). The ced-3/ICE-like protease MCH2 is activated during apoptosis and cleaves the death substrate lamin-A. J. Biol. Chem. *271*, 16443-16446.

Ortiz, B.D., Nelson, P.J., and Krensky, A.M. (1997). Switching gears during T-cell maturation: RANTES and late transcription. Immunol. Today *18*, 468-471.

Oshimi, Y. and Miyazaki, S. (1995). Fas antigen-mediated DNA fragmentation and apoptotic morphologic change are regulated by elevated cytosolic Ca^{2+} level. J. Immunol. *154*, 599-609.

Owen-Schaub, L.B., Yonehara, S., Crump, W.L., and Grimm, E.A. (1992). DNA fragmentation and cell-death is selectively triggered in activated human-lymphocytes by Fas antigen engagement. Cell. Immunol. *140*, 197-205.

Pages, F., Ragueneau, M., Rottapel, R., Truneh, A., Nunes, J., Imbert, J., and Olive, D. (1994). Binding of phosphatidylinositol-3 kinase to CD28 is required for T cell signalling. Nature *369*, 327-329.

Pan, G.H., Orourke, K., Chinnaiyan, A.M., Gentz, R., Ebner, R., Ni, J., and Dixit, V.M. (1997). The receptor for the cytotoxic ligand TRAIL. Science *276*, 111-113.

Papoff, G., Cascino, I., Eramo, A., Starace, G., Lynch, D.H., and Ruberti, G. (1996). An N-terminal domain shared by Fas/Apo-1 (CD95) soluble variants prevents cell-death *in-vitro*. J. Immunol. *156*, 4622-4630.

Park, D.J., Rho, H.W., and Rhee, S.G. (1991). CD3 stimulation causes phosphorylation of phospholipase C-γ-1 on serine and tyrosine residues in a human T-cell line. Proc. Natl. Acad. Sci. U.S.A. *88*, 5453-5456.

Pawelec, G., Sansom, D., Rehbein, A., Adibzadeh, M., and Beckman, I. (1996). Decrease proliferative capacity and increased susceptibility to activation induced cell death in late passage human CD4+ TCR2+ cultured T cell clones. Exp. Gerontol. *31*, 655-668.

Pearson, T.C., Alexander, D.Z., Winn, K.J., Linsley, P.S., Lowry, R.P., and Larsen, C.P. (1994). Transplantation tolerance induced by CTLA-4-Ig. Trans. *57*, 1701-1706.

Perlmutter, R.M., Levin, S.D., Appleby, M.W., Anderson, S.J., and Alberolaila, J. (1993). Regulation of lymphocyte function by protein-phosphorylation. Ann. Rev. Immunol. *11*, 451-499.

Perrin, P.J., Scott, D., Quigley, L., Albert, P.S., Feder, O., Gray, G.S., Abe, R., June, C.H., and Racke, M.K. (1995). Role of B7/ CD28 CTLA-4 in the induction of chronic relapsing experimental allergic encephalomyelitis. J. Immunol. *154*, 1481-1490.

Perrin, P.J., Maldonado, J.H., Davis, T.A., June, C.H., and Racke, M.K. (1996). CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. J. Immunol. *157*, 1333-1336.

Peter, M.E., Kischkel, F.C., Scheuerpflug, C.G., Medema, J.P., Debatin, K.M., and Krammer, P.H. (1997). Resistance of cultured peripheral T cells towards activation-induced cell death involves a lack of recruitment of FLICE

(MACH/caspase 8) to the CD95 death-inducing signaling complex. Eur. J. Immunol. *27*, 1207-1212.

Peterson, M. and Miller, J. (1990). Invariant chain influences the immunological recognition of class II molecules. Nature *345*, 172-174.

Piazza, C., Montani, M.S.G., Moretti, S., Cundari, E., and Piccolella, E. (1997). CD4+ T cells kill CD8+ T cells via Fas/Fas ligand-mediated apoptosis. J. Immunol. *158*, 1503-1506.

Pichler, W.J. and Wyss-Coray, T. (1994). T cells as antigen presenting cells. Immunol. Today *15*, 312-315.

Poulton, K.V., Hajeer, A., Hillarby, M.C., Ollier, W.E.R., and Holt, P.J.L. (1995). Alternatively spliced Fas messenger-RNA in SLE. Arthritis Rheum. *38*, 681.

Prabhu Das, M.R., Zamvil, S.S., Weiner, H.L., Sharpe, A.H., and Kuchroo, V.K. (1995). Reciprocal expression of costimulatory molecules B7-1 and B7-2 on murine T cells following activation. Eur. J. Immunol. *25*, 207-211.

Prasad, K.V.S., Cai, Y., Raab, M., Duckworth, B., Cantley, L., Schoelson, S.E., and Rudd, C.E. (1994). T cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(p)-met-Xaa-Met motif. Proc. Natl. Acad. Sci. U.S.A. *91*, 2834-2838.

Pullen, A.M., Kappler, J.W., and Marrack, P. (1989). Tolerance to self antigens shapes the T-cell repertoire. Immunol. Rev. *107*, 125-139.

Raab, M., Cai, Y.C., Bunnell, S.C., Heyeck, S., Berg, L.J., and Rudd, C.E. (1995). p56*lck* and p59*fyn* regulate CD28 binding to PI3 Kinase, growth factor receptor bound GRB-2 and T cell specific PTK, *ITK*: Implications for T cell costimulation. Proc. Natl. Acad. Sci. U.S.A. *92*, 8891-8895.

Racke, M.K., Scott, D.E., Quigley, L., Gray, G.S., Abe, R., June, C.H., and Perrin, P.J. (1995). Distinct roles for B7-1 (CD-80) and B7-2 (CD-86) in the initiation of experimental allergic encephalomyelitis. J. Clin. Invest. *96*, 2195-2203.

Radvanyi, L.G., Shi, Y., Vaziri, H., Sharma, A., Dhala, R., Mills, G., and Miller, R.G. (1996). CD28 costimulation inhibits TCR induced apoptosis during a primary T cell response. J. Immunol. *156*, 1788-1798.

Ramensee, H.G., Kroschewski, R., and Frangoulis, B. (1989). Clonal anergy induced in mature Vβ6$^+$ T lymphocytes on immunising Mls-1^b mice with Mls-1^a expressing cells. Nature *339*, 541-545.

Ramsdell, F., Seaman, M.S., Miller, R.E., Picha, K.S., Kennedy, M.K., and Lynch, D.H. (1994a). Differential ability of Th1 and Th2 T-cells to express Fas ligand and to undergo activation-induced cell death. Int. Immunol. *6*, 1545-1553.

Ramsdell, F., Seaman, M.S., Miller, R.E., Tough, T.W., Alderson, M.R., and Lynch, D.H. (1994b). *gld/gld* mice are unable to express a functional ligand for Fas. Eur. J. Immunol. *24*, 928-933.

Rathmell, J.C., Cooke, M.P., Ho, W.Y., Grein, J., Townsend, S.E., Davis, M.M., and Goodnow, C.C. (1995). CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. Nature *376*, 181-184.

Rathmell, J.C. and Goodnow, C.C. (1995). Autoimmunity - the Fas track. Curr. Biol. *5*, 1218-1221.

Razi-Wolf, Z., Freeman, G.J., Galvin, F., Benacerraf, B., Nadler, L., and Reiser, H. (1992). Expression and function of the murine B7 antigen, the major costimulatory molecule expressed on peritoneal exudate cells. Proc. Natl. Acad. Sci. U.S.A. *89*, 4210-4214.

Reiner, S.L. and Locksley, R.M. (1993). Cytokines in the differentiation of Th1/Th2 CD4+ subsets in leishmaniasis. J. Cell. Biochem. *53*, 323-328.

Reinhold, M.I., Lindberg, F.P., Kersh, G.J., Allen, P.M., and Brown, E.J. (1997). Costimulation of T cell activation by integrin-associated protein (CD47) is an adhesion-dependent, CD28-independent signaling pathway. J. Exp. Med. *185*, 1-11.

Rodriguez-Tarduchy, G. and Lopez-Rivas, A. (1989). Phorbol esters inhibit apoptosis in IL-2-dependent T lymphocytes. Biochem. Biophys. Res. Comm. *164*, 1069-1075.

Romagnani, S. (1997). The Th1/Th2 paradigm. Immunol. Today *18*, 263-266.

Rothstein, T.L., Wang, J.K.M., Panka, D.J., Foote, L.C., Wang, Z., Stanger, B., Cui, H., Ju, S., and Marshak-Rothstein, A. (1995). Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. Nature *374*, 163-165.

Rouvier, E., Luciani, M-F., and Golstein, P. (1993). Fas involvement in Ca^{2+}-independent T cell-mediated cytotoxicity. J. Exp. Med. *177*, 195-200.

Rudd, C.E., Janssen, O., Cai, Y.C., Dasilva, A.J., Raab, M., and Prasad, K.V.S. (1994). Two-step TCR-ζ/CD3-CD4 and CD28 signaling in T-cells - SH2/SH3 domains, protein-tyrosine and lipid kinases. Immunol. Today *15*, 225-234.

Russell, J.H, White, C.L, Loh, D.Y, and Meleedy-Rey, P. (1991). Receptor-stimulated death pathway is opened by antigen in mature T cells. Proc. Natl. Acad. Sci. U.S.A. *88*, 2151-2155.

Russell, J.H., Rush, B., Abrahams, S.I., and Wang, R. (1992). Sensitivity to anti-CD3-stimulated suicide is independent of functional phenotype. Eur. J. Immunol. *22*, 1655-1658.

Russell, J.H., Rush, B., Weaver, C., and Wang, R. (1993). Mature T cells of autoimmune lpr/lpr mice have a defect in antigen-stimulated suicide. Proc. Natl. Acad. Sci. U.S.A. *90*, 4409-4413.

Samelson, L.E. and Klausner, R.D. (1992). Tyrosine kinases and tyrosine-based activation motifs - current research on activation via the T-cell antigen receptor. J. Biol. Chem. *267*, 24913-24916.

Sansom, D.M and Hall, N.D (1993). B7/BB1, the ligand for CD28 is expressed on repeatedly activated human T cells *in vitro*. Eur. J. Immunol. *23*, 295-298.

Sansom, D.M., Wilson, A., Boshell, M., Lewis, J., and Hall, N.D (1993). B7/CD28 but not LFA-3/CD2 interactions can provide third party costimulation for human T cell activation. Immunol. *80*, 242-247.

Sarin, A., Wu, M.L., and Henkart, P.A. (1996). Different interleukin-1β converting enzyme (ICE) family protease requirements for the apoptotic death of T lymphocytes triggered by diverse stimuli. J. Exp. Med. *184*, 2445-2450.

Sato, T., Irie, S., Kitada, S., and Reed, J.C. (1995). FAP-1 a protein tyrosine phosphatase that associates with Fas. Science *268*, 411-415.

Schraven, B. and Peter, M.E. (1995). Apo-1(CD95)-mediated apoptosis in Jurkat cells does not involve *src* kinases or CD45. FEBS Lett. *368*, 491-494.

Schultz-Osthoff, K., Krammer, P.H., and Droge, W. (1994). Divergent signalling via APO/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. EMBO J. *13*, 4587-4596.

Schwartz, L.M. and Osborne, B.A. (1994). Ced-3/ICE - evolutionarily conserved regulation of cell-death. BioEssays *16*, 387-389.

Schwartz, R.H. (1990). A cell culture model for T lymphocyte clonal anergy. Science *248*, 1349-1356.

Seder, R.A., Gazzinelli, R., Sher, A., and Paul, W.E. (1993). Interleukin-12 acts directly on CD4+ T-cells to enhance priming for interferon-γ production and diminishes interleukin-4 inhibition of such priming. Proc. Natl. Acad. Sci. U.S.A. *90*, 10188-10192.

Seed, B. (1987). An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature *329*, 840-842.

Sheridan, J.P., Marsters, S.A., Pitti, R.M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C.L., Baker, K., Wood, W.I., Goddard, A.D., Godowski, P., and Ashkenazi, A. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science *277*, 818-821.

Shi, Y., Glynn, J., Guilbert, L.J., Cotter, T.G., Bisonette, R.P., and Green, D.R. (1992). Role of c-myc in activation-induced apoptotic cell death in T-cell hybridoma. Science *257*, 212.

Shimizu, S., Eguchi, Y., Kamiike, W., Waguri, S., Uchiyama, Y., Matsuda, H., and Tsujimoto, Y. (1996). BCL-2 blocks loss of mitochondrial-membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory-chain inhibitors. Oncogene *13*, 21-29.

Shiratori, T., Miyatake, S., Ohno, H., Nakaseko, C., Isono, K., Bonifacino, J.S., and Saito, T. (1997). Tyrosine phosphorylation controls internalization of CTLA-4

by regulating its interaction with clathrin-associated adaptor complex AP-2. Immunity *6*, 583-589.

Shuford, W.W., Klussman, K., Tritchler, D.D., Loo, D.T., Chalupny, J., Siadak, A.W., Brown, T.J., Emswiler, J., Raecho, H., Larsen, C.P., Pearson, T.C., Ledbetter, J.A., Aruffo, A., and Mittler, R.S. (1997). 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification *in vivo* of cytotoxic T cell responses. J. Exp. Med. *186*, 47-55.

Sidhu, S., Bal, V., Batchelor, J.R., Lombardi, G., and Lechler, R.I. (1992). Human T cells cannot act as autonomous antigen presenting cells but induce tolerance in antigen specific and alloreactive responder cells. J. Exp. Med. *176*, 875-880.

Sidman, C., Marshall, N.D., and von Boehmer, H. (1992). Transgenic T cell receptor interactions in the lymphoproliferative and autoimmune syndromes of the lpr and gld mutant mice. Eur. J. Immunol. *22*, 499-504.

Simonian, P.L., Grillot, D.A.M., and Nunez, G. (1997). BAK can accelerate chemotherapy-induced cell death independently of its heterodimerization with BCL-X_L and BCL-2. Oncogene *15*, 1871-1875.

Singer, G.G. and Abbas, A.K. (1994). The Fas antigen is involved in peripheral but not thymic deletion of T-lymphocytes in T-cell receptor transgenic mice. Immunity *1*, 365-371.

Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J., and Owen, J.J.T. (1989). Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T-cells in thymic cultures. Nature *337*, 181-184.

Smith, C.A., Farrah, T., and Goodwin, R.G (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. Cell *76*, 959-962.

Smyth, M.J., Perry, D.K., Zhang, J.D., Poirier, G.G., Hannun, Y.A., and Obeid, L.M. (1996). PRICE - a downstream target for ceramide-induced apoptosis and for the inhibitory-action of BCL-2. Biochem. J *316*, 25-28.

Sperling, A.I., Auger, J.A., Ehst, B.D., Rulifson, I.C., Thompson, C.B., and Bluestone, J.A. (1996). CD28/B7 interactions deliver a unique signal to naive T

cells that regulates cell survival but not early proliferation. J. Immunol. *157*, 3909-3917.

Sprent, J., Tough, D.F., and Sun, S.Q. (1997). Factors controlling the turnover of T memory cells. Immunol. Rev. *156*, 79-85.

Stack, R.M., Lenschow, D.J., Gray, G.S., Bluestone, J.A., and Fitch, F.W. (1994). IL-4 treatment of small splenic B-cells induces costimulatory molecules B7-1 and B7-2. J. Immunol. *152*, 5723-5733.

Stanger, B.Z., Leder, P., Lee, T.H., Kim, E., and Seed, B. (1995). RIP - a novel protein containing a death domain that interacts with Fas/Apo-1 (CD95) in yeast and causes cell-death. Cell *81*, 513-523.

Strasser, A., Harris, A.W., Huang, D.C.S., Krammer, P.H., and Cory, S. (1995). BCL-2 and Fas/Apo-1 regulate distinct pathways to lymphocyte apoptosis. EMBO J. *14*, 6136-6147.

Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. (1994). JNK is involved in signal integration during costimulation of T lymphocytes. Cell *77*, 727-736.

Su, X., Zhou, T., Wang, Z., Yang, P.A., Jope, R.S., and Mountz, J.D. (1995). Defective expression of hematopoietic-cell protein-tyrosine- phosphatase (HCP) in lymphoid-cells blocks Fas-mediated apoptosis. Immunity *2*, 353-362.

Suda, T. and Nagata, S. (1994). Purification and characterisation of the Fas-ligand that induces apoptosis. J. Exp. Med. *179*, 873-879.

Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1994). Molecular cloning and expression of the Fas ligand, a novel member of the Tumour Necrosis Factor family. Cell *75*, 1169-1178.

Suda, T., Okazaki, T., Naito, Y., Yokota, T., Arai, N., Ozaki, S., Nakao, K., and Nagata, S. (1995). Expression of the Fas ligand in cells of T-cell lineage. J. Immunol. *154*, 3806-3813.

Suda, T., Masato, T., Keiko, M., and Nagata, S. (1996). Apoptosis of mouse naive T cells induced by recombinant soluble Fas Ligand and activation-induced resistance to Fas ligand. J. Immunol. *157*, 3918-3924.

Susin, S.A., Zamzami, N., Castedo, M., Daugas, E., Wang, H.G., Geley, S., Fassy, F., Reed, J.C., and Kroemer, G. (1997). The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/Apo-1/CD95- and ceramide- induced apoptosis. J. Exp. Med. *186*, 25-37.

Suzuki, H., Kundig, T.M., Furlonger, C., Wakeham, A., Timms, E., Matsuyama, T., Schmits, R., Simard, J.J.L., Ohashi, P.S., Griesser, H., Taniguchi, T., Paige, C.J., and Mak, T.W. (1995). Deregulated T-cell activation and autoimmunity in mice lacking interleukin-2 receptor-β. Science *268*, 1472-1476.

Takahashi, S., Maecker, H.T., and Levy, R. (1989). DNA fragmentation and cell-death mediated by T-cell antigen receptor CD3 complex on a leukemia T-cell line. Eur. J. Immunol. *19*, 1911-1919.

Takahashi, T., Tanaka, M., Brannan, C.I, Jenkins, N.A., Copeland, N.G., Suda, T., and Nagata, S. (1994a). Generalised lymphoproliferative disease in Mice caused by a point mutation in the Fas ligand. Cell *76*, 969-976.

Takahashi, T., Tanaka, M., Inazawa, J., Abe, T., Suda, T., and Nagata, S. (1994b). Human Fas ligand - gene structure, chromosomal location and species- specificity. Int. Immunol. *6*, 1567-1574.

Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J.A., and Reed, J.C. (1995). Cloning and functional-analysis of BAG-1 - a novel BCL-2-binding protein with anti-cell death activity. Cell *80*, 279-284.

Tan, R.S., Teh, S.J., Ledbetter, J.A., Linsley, P.S., and Teh, H.S. (1992). B7 costimulates proliferation of CD4-8+ T lymphocytes but is not required for the deletion of immature CD4+8+ thymocytes. J. Immunol. *149*, 3217-3224.

Tanaka, M., Suda, T., Takahashi, T., and Nagata, S. (1995). Expression of the functional soluble form of human Fas ligand in activated lymphocytes. EMBO J. *14*, 1129-1135.

Tanaka, M., Suda, T., Haze, K., Nakamura, N., Sato, K., Kimurak, F., Motoyashi, K., Mizuki, M., Tagawa, S., Ohga, S., Hatake, K., Drummond, A., and Nagata, S. (1996). Fas ligand in human serum. Nature Med. *2*, 317-322.

Taupin, J.L., Tian, Q.S., Kedersha, N., Robertson, M., and Anderson, P. (1995). The RNA-binding protein TIAR is translocated from the nucleus to the cytoplasm during Fas-mediated apoptotic cell-death. Proc. Natl. Acad. Sci. U.S.A. *92*, 1629-1633.

Tepper, C.G., Jayadev, S., Liu, B., Bielawska, A., Wolff, R., Yonehara, S., Hannun, Y.A., and Seldin, M.F. (1995). Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. Proc. Natl. Acad. Sci. U.S.A. *92*, 8443-8447.

Thome, M., Duplay, P., Guttinger, M., and Acuto, O. (1995). Syk and ZAP70 mediate recruitment of p56^{lck}/ CD4 to the activated TCR/ CD3ζ complex. J. Exp. Med. *181*, 1997-2006.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J.L., Schroter, M., Scaffidi, C., Krammer, P.H., Peter, M.E., and Tschopp, J. (1997). Viral FLICE-inhibitory proteins (FLIPS) prevent apoptosis induced by death receptors. Nature *386*, 517-521.

Thompson, C., Lindsten, T., Ledbetter, J., Kunkel, S., Young, H., Emerson, S., Leiden, J., and June, C. (1993). CD28 activation pathway regulates the production of multiple T cell-derived Lymphokines/cytokines. Proc. Natl. Acad. Sci. U.S.A. *86*, 1333-1337.

Tian, Q.S., Taupin, J.L., Elledge, S., Robertson, M., and Anderson, P. (1995). Fas-activated serine threonine kinase (FAST) phosphorylates TIA-1 during Fas-mediated apoptosis. J. Exp. Med. *182*, 865-874.

Timson-Gauen, L.K., Zhu, Y., Letourneur, F., Hu, Q., Bolen, J.B., Matis, L.A., Klausner, R.D., and Shaw, A.S. (1994). Interactions of p59^{fyn} and ZAP70 with TCR activation motifs:- Defining the nature of a signalling motif. Mol. Cell. Biol. *14*, 3729-3741.

Tivol, E.A., Borriello, F., Schweitzer, A.N., Lynch, W.P., Bluestone, J.A., and Sharpe, A.H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity *3*, 541-547.

Tivol, E.A., Boyd, S.D., McKeon, S., Borriello, F., Nickerson, P., Strom, T.B., and Sharpe, A.H. (1997). CTLA-4 - Ig prevents lymphoproliferation and fatal

multiorgan tissue destruction in CTLA-4-deficient mice. J. Immunol. *158*, 5091-5094.

Tomei, L.D., Kanter, P., and Wenner, C.E. (1988). Inhibition of radiation-induced apoptosis *in vitro* by tumor promoters. Biochem. Biophys. Res. Comm. *155*, 324-331.

Townsend, A. and Bodmer, H. (1989). Antigen recognition by class-I restricted T lymphocytes. Ann. Rev. Immunol. *7*, 601-624.

Trauth, B.C., Klas, C., Peters, A.M.J., Matzku, S., Moller, P., Falk, W., Debatin, K.M., and Krammer, P.H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science *245*, 301.

Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A., and Kelly, A. (1991). Sequences encoded in the class II region of the MHC related to the "ABC" superfamily of transporters. Nature *348*, 741-743.

Tsuruta, L., Lee, H.J., Masuda, E.S., Koyano-Nakagawa, N., Arai, K., and Yokota, T. (1995). Cyclic AMP inhibits expression of the IL-2 gene through the NFAT site and transfection of NFAT cDNAs abrogate the sensitivity of EL-4 cells to cyclic AMP. J. Immunol. *154*, 5255-5264.

Tucek-Szabo, C.L., Andjelic, S., Lacy, E., Elkon, K.B., and Nikoliczugic, J. (1996). Surface T-cell Fas receptor CD95 regulation, *in-vivo* activation, and apoptosis - activation-induced death can occur without Fas receptor. J. Immunol. *156*, 192-200.

Ucker, D.S., Meyers, J., and Obermiller, P.S. (1992). Activation-driven T-cell death .2: Quantitative differences alone distinguish stimuli triggering nontransformed T-cell proliferation or death. J. Immunol. *149*, 1583-1592.

Ulrich, E., Kauffmann-Zeh, A., Hueber, A.O., Williamson, J., Chittenden, T., Ma, A., and Evan, G. (1997). Gene structure, cDNA sequence, and expression of murine BAK, a pro-apoptotic BCL-2 family member. Genomics *44*, 195-200.

van der Merwe, P.A., Bodian, D.L., Daenke, S., Linsley, P., and Davis, S.J. (1997). CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. J. Exp. Med. *185*, 393-403.

van Parijs, L., Ibraghimov, A., and Abbas, A.K. (1996). The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. Immunity *4*, 321-328.

van Seventer, G.A, Shimizu, Y., Horgan, K.J, and Shaw, S. (1990). The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. J. Immunol. *144*, 4579-4586.

Vandenberghe, P.A. and Ceuppens, J.L. (1990). Flow cytometric measurement of cytoplasmic free calcium in human peripheral-blood lymphocytes-T with fluo-3, a new fluorescent calcium indicator. J. Immunol. Meths. *127*, 197-205.

Vaux, D.L., Haecker, G., and Strasser, A. (1994). An evolutionary perspective on apoptosis. Cell *76*, 777-779.

Vella, A.T., Mitchell, T., Groth, B., Linsley, P.S., Green, J.M., Thompson, C.B., Kappler, J.W., and Marrack, P. (1997). CD28 engagement and proinflammatory cytokines contribute to T cell expansion and long-term survival in vivo. J. Immunol. *158*, 4714-4720.

Vergelli, M., Hemmer, B., Muraro, P.A., Tranquill, L., Biddison, W.E., Sarin, A., McFarland, H.F., and Martin, R. (1997). Human autoreactive CD4+ T cell clones use perforin- or Fas/Fas ligand-mediated pathways for target cell lysis. J. Immunol. *158*, 2756-2761.

Via, C.S., Shustov, A., Nguyen, P., Drappa, J., and Elkon, K. (1995). Fas ligand deficient donor T-cells fail to produce acute graft- versus-host disease (GVHD) and instead induce lupus-like autoimmunity in the parent-into-F1 (p-F1) model. Arthritis Rheum. *38*, 872.

Via, C.S., Nguyen, P., Shustov, A., Drappa, J., and Elkon, K.B. (1996). A major role for the Fas pathway in acute graft-versus-host disease. J. Immunol. *157*, 5387-5393.

Vignaux, F., Vivier, E., Malissen, B., Depraetere, V., Nagata, S., and Golstein, P. (1995). TCR/CD3 coupling to Fas-based cytotoxicity. J. Exp. Med. *181*, 781-786.

Vincenz, C. and Dixit, V.M. (1997). Fas-associated death domain protein interleukin-1β-converting enzyme 2 (FLICE 2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. J. Biol. Chem. *272*, 6578-6583.

Vindelov, L.L., Christensen, I.J., and Nissen, N.I. (1983). Standardization of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red-blood-cells as internal reference-standards. Cytometry *3*, 328-331.

Waldmann, H., Cobbold, S., Lefkovits, I., and Klaus, G.G.B. (1987). Lymphocytes: a practical approach (Oxford: IRL Press).

Walunas, T.L., Lenschow, D.J., Bakker, C.Y., Linsley, P.S., Freeman, G.J., Green, J.M., Thompson, C.B., and Bluestone, J.A (1994). CTLA-4 can function as a negative regulator of T cell activation. Immunity *1*, 405-413.

Walunas, T.L., Bakker, C.Y., and Bluestone, J.A. (1996). CTLA-4 ligation blocks CD28-dependent T cell activation. J. Exp. Med. *183*, 2541-2550.

Wang, H., Balderas, R., Rosenberg, J., Huang, E.C.M., and Chen, Z. (1996a). Expression and function of CTLA-4 on human T-cells. Tissue antigens *48*, TC203.

Wang, R.D., Rogers, A.M., Rush, B.J., and Russell, J.H. (1996b). Induction of sensitivity to activation-induced death in primary CD4(+) cells - a role for interleukin-2 in the negative regulation of responses by mature CD4(+) T-cells. Eur. J. Immunol. *26*, 2263-2270.

Wang, Z.Q., Stingl, L., Morrison, C., Jantsch, M., Los, M., Schulzeosthoff, K., and Wagner, E.F. (1997). PARP is important for genomic stability but dispensable in apoptosis. Gene Devel. *11*, 2347-2358.

Ward, S., Westwick, J., Hall, N., and Sansom, D. (1993). CD28 ligation elevates PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in T cells. Eur. J. Immunol. *23*, 2572-2577.

Ward, S.G., Wilson, A., Turner, L., Westwick, J., and Sansom, D.M. (1995). Inhibition of CD28-mediated T cell costimulation by the phosphoinositide 3-Kinase inhibitor wortmannin. Eur. J. Immunol. *25*, 526-532.

Watanabe, N., Arase, H., Kurasawa, K., Iwamoto, I., Kayagaki, N., Yagita, H., Okumura, K., Miyatake, S., and Saito, T. (1997). Th1 and Th2 subsets equally undergo Fas-dependent and -independent activation-induced cell death. Eur. J. Immunol. *27*, 1858-1864.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature *356*, 314-317.

Waterhouse, P., Penninger, J.M., Timms, E., Wakeham, A., Shahinian, A., Lee, K.P., Thompson, C.B., Griesser, H., and Mak, T.W. (1995). Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. Science *270*, 985-988.

Webb, L.M.C., Walmsley, M.J., and Feldmann, M. (1996). Prevention and amelioration of collagen induced arthritis by blockade of the CD28 co-stimulatory pathway: requirement for both B7-1 and B7-2. Eur. J. Immunol. *26*, 2320-2328.

Weber, G.F., Abromsonleeman, S., and Cantor, H. (1995). A signaling pathway coupled to T-cell receptor ligation by MMTV superantigen leading to transient activation and programmed cell- death. Immunity *2*, 363-372.

Weiss, A., Imboden, J., Shoback, D., and Stobo, J. (1984). Role of T3 surface molecules in human T-cell activation - T3- dependent activation results in an increase in cytoplasmic free calcium. Proc. Natl. Acad. Sci. U.S.A. *81*, 4169-4173.

Wesselborg, S., Janssen, O., and Kabelitz, D. (1993). Induction of activation-driven death (apoptosis) in activated but not resting peripheral-blood T-cells. J. Immunol. *150*, 4338-4345.

Westwick, J.K., Bielawska, A.E., Dbaibo, G., Hannun, Y.A., and Brenner, D.A. (1995). Ceramide activates the stress-activated protein kinases. J. Biol. Chem. *270*, 22689-22692.

White, K., Tahaoglu, E., and Steller, H. (1996). Cell-killing by the *Drosophila* gene reaper. Science *271*, 805-807.

Wiley, S.R., Schooley, K., Smolak, P.J., Din, W.S., Huang, C.P., Nicholl, J.K., Sutherland, G.R., Smith, T.D., Rauch, C., Smith, C.A., and Goodwin, R.G. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity *3*, 673-682.

Wilson, D.J., Fortner, K.A., Lynch, D.H., Mattingly, R.R., Macara, I.G., Posada, J.A., and Budd, R.C. (1996). JNK, but not MAPK, activation is associated with Fas-mediated apoptosis in human T-cells. Eur. J. Immunol. *26*, 989-994.

Wong, G.H.W. and Goeddel, D.V. (1994). Fas antigen and p55 TNF receptor signal apoptosis through distinct pathways. J. Immunol. *152*, 1751-1755.

Woronicz, J.D., Calnan, B., Ngo, V., and Winoto, A. (1994). Requirement for the orphan steroid receptor Nur77 in apoptosis of T cell hybridomas. Nature *367*, 277.

Wright, S.C., Kumar, P., Tam, A.W., Shen, N.P., Varma, M., and Larrick, J.W. (1992). Apoptosis and DNA fragmentation precede TNF-induced cytolysis in U937 cells. J. Cell. Biochem. *48*, 344-355.

Wu, D.Y., Wallen, H.D., Inohara, N., and Nunez, G. (1997a). Interaction and regulation of the *Caenorhabditis elegans* death protease ced-3 by ced-4 and ced-9. J. Biol. Chem. *272*, 21449-21454.

Wu, D.Y., Wallen, H.D., and Nunez, G. (1997b). Interaction and regulation of subcellular localisation of ced-4 by ced-9. Science *275*, 1126-1129.

Wu, J., Zhou, T., He, J., and Mountz, J.D. (1993). Autoimmune disease in mice due to integration of an endogenous retrovirus in an apoptosis gene. J. Exp. Med. *178*, 461-468.

Wu, J., Zhou, T., Zhang, J., He, H., Gause, W.C., and Mountz, J.D (1994). Correction of accelerated autoimmune disease by early replacement of the mutated *lpr* gene with the normal *Fas* apoptosis gene in the T cells of transgenic MRL-*lpr/lpr* mice. Proc. Natl. Acad. Sci. U.S.A. *91*, 2344-2348.

Yang, J., Liu, X., Bhalla, K., Naekyung, K., Ibrado, A.M., Cai, J., Peng, T.-I., Jones, D.P., and Wang, X. (1997a). Prevention of apoptosis by BCL-2: release of cytochrome c from mitochondria blocked. Science *275*, 1129-1132.

Yang, X.L., Khosravi-Far, R., Chang, H.Y., and Baltimore, D. (1997b). DAXX, a novel Fas-binding protein that activates JNK and apoptosis. Cell *89*, 1067-1076.

Yang, Y., Mercep, M., Ware, C.F., and Ashwell, J.D. (1995). Fas and activation-induced Fas-ligand mediate apoptosis of T cell hybridomas: inhibition of Fas-ligand expression by retinoic acid and glucocorticoids. J. Exp. Med. *181*, 1673-1682.

Yewdell, J.W. and Bennick, J.R. (1992). Cell biology of antigen processing and presentation to MHC class I molecule-restricted T lymphocytes. Adv. Immunol. *52*, 1-123.

Yonehara, S., Ishii, A., and Yonehara, M. (1989). A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J. Exp. Med. *169*, 1747.

Yuan, J.Y. and Horvitz, H.R. (1990). The *Caenorhabditis-elegans* genes *ced-3* and *ced-4* act cell autonomously to cause programmed cell-death. Devel. Biol. *138*, 33-41.

Yuan, J.Y. and Horvitz, H.R. (1992). The *Caenorhabditis-elegans* cell-death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell-death. Devel. *116*, 309-320.

Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S.A., Petit, P.X., Mignotte, B., and Kroemer, G. (1995a). Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell-death. J. Exp. Med. *182*, 367-377.

Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J.L., Petit, P.X., and Kroemer, G. (1995b). Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death *in-vivo*. J. Exp. Med. *181*, 1661-1672.

Zamzami, N., Susin, S.A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M., and Kroemer, G. (1996). Mitochondrial control of nuclear apoptosis. J. Exp. Med. *183*, 1533-1544.

Zehava, C.L. and Cleary, M.L. (1990). Membrane topology of the BCL-2 proto-oncogenic protein demonstrated *in vitro*. J. Biol. Chem. *265*, 4929-4933.

Zha, J.P., Harada, H., Osipov, K., Jockel, J., Waksman, G., and Korsmeyer, S.J. (1997). BH3 domain of BAD is required for heterodimerization with BCL-X_L and pro-apoptotic activity. J. Biol. Chem. *272*, 24101-24104.

Zhang, X.H., Brunner, T., Carter, L., Dutton, R.W., Rogers, P., Bradley, L., Sato, T., Reed, J.C., Green, D., and Swain, S.L. (1997). Unequal death in T helper cell

Th1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. J. Exp. Med. *185*, 1837-1849.

Zheng, L.X., Fisher, G., Miller, R.E., Peschon, J., Lynch, D.H., and Lenardo, M.J. (1995). Induction of apoptosis in mature T-cells by tumor-necrosis-factor. Nature *377*, 348-351.

Zhou, T., Bluethmann, H., Zhang, J., Edwards III, K., and Mountz, J.D. (1992). Defective maintenance of T cell tolerance to a superantigen in MRL *lpr/lpr* mice. J. Exp. Med. *176*, 1063-1060.

Zhu, L. and Anasetti, C. (1995). Cell cycle control of apoptosis in human leukemic T cells. J. Immunol. *154*, 192-200.

Zinkernagel, R.M., and Doherty, P.C. (1974). Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogenic system. Nature *248*, 710-702

Zipp, F., Martin, R., Lichtenfels, R., Roth, W., Dichgans, J., Krammer, P.H., and Weller, M. (1997). Human autoreactive and foreign antigen-specific T cells resist apoptosis induced by soluble recombinant CD95 ligand. J. Immunol. *159*, 2108-2115.

Appendix 1: Suppliers

(A) Suppliers of Reagents

AB serum (human)	National Blood Service
Apoptest™	Boehringer-Mannheim
Aquaphenol	Appligene
BB2116 (metalloproteinase inhibitor)	kind gift from British Biotechnology
DE81 discs and blotting paper	Whatman Scientific
DNA markers (ØX174)	NBL Gene Sciences Ltd
Dynabeads (Ab-coated)	Dynal (UK) Ltd
Fix and Perm kit	TCS Biologicals Ltd
Fluo-3 / acetoxymethyl ester	Calbiochem (UK) Ltd
Glass fibre filter mats	Skatron Ltd
Heparin (Monoparin)	CP Pharmaceuticals Ltd
Hybond-N membrane	Hybaid Ltd
IL-2	kind gift from Glaxo Ltd
Ionomycin	Calbiochem (UK) Ltd
L-glutamine	Life Technologies
Lymphoprep	Nycomed (UK) Ltd
Mycoplasma ELISA kit	Boehringer Mannheim

Optiscint HiSafe	Wallac Ltd
α^{32}P dCTP	Amersham International
Phenol (water saturated)	Appligene
Phosphate-buffered saline tablets	Oxoid
Penicillin/Streptomycin	Life Technologies
Prime-a-gene kit	Promega
Pyruvate	Life Technologies
TUNEL kit	Boehringer Mannheim
Tritiated thymidine	Amersham International
Trypsin-EDTA	Life Technologies
Z-VAD-FMK	Enzyme Systems Products

(B) Suppliers Contact Numbers

The following list states the location and telephone numbers of the specialist suppliers of reagents.

Amersham International plc (Aylesbury, UK)	01494 544000
Appligene (Britley, UK)	0191 4920022
Becton Dickinson (UK) Ltd (Oxford, UK)	01865 748844
Boehringer Mannheim (Lewes, UK)	01273 480444
British Biotechnology (Oxford, UK)	01865 780800
Calbiochem (UK) Ltd (Nottingham, UK)	0115 943 0840
CP Pharmaceuticals Ltd (Wrexham, UK) (Distributed via Fisons, Leicester, UK)	
Dynal (UK) Ltd (Bromborough, UK)	0151 3461234
Enzyme Systems Products (California, USA) (Contact via internet web page)	
Fahrenheit Laboratory Supplies (Bristol, UK)	0117 9701667
Hybaid Ltd (Teddington, UK)	0181 614 1000
Life Technologies (Paisley, UK)	0141 8146100
National Blood Service (Bristol, UK)	0117 9112168
NBL Gene Sciences Ltd (Cramlington, UK)	01670 732992
Nycomed (UK) Ltd (Birmingham, UK)	0121 7422444

Oxoid Ltd (Basingstoke, UK) (Distributed via Farenheit Laboratory Supplies, Bristol, UK)	
Pharmacia Biotech Ltd (St Albans, UK)	01727 814000
Promega Ltd (Southampton, UK)	01703 760225
R&D Systems Europe Ltd (Abingdon, UK)	01235 529449
Serotec Ltd (Oxford, UK)	01865 373899
Sigma Aldrich Ltd (Poole, UK)	01202 733114
Skatron Ltd (Newmarket, UK)	01638 660600
TCS Biologicals Ltd (Buckingham, UK)	01296 714071
Wallac Ltd (Milton Keynes, UK) (Distributed through Fisons and Pharmacia Biotech)	
Whatman Scientific (Maidstone, UK)	01622 692022

Appendix 2: Media

(A) Dulbecco's Minimal Essential Medium (DMEM) (pH 7.4)

The following were added to 400ml of autoclaved ddH2O:-

DMEM (10X)	55ml
FCS	50ml
Penicillin / Streptomycin (10000µg/ml / 10000IU/ml)	5ml
Sodium bicarbonate	28ml (0.4% final)
Sodium pyruvate	5ml (1mM final)
Nucleosides (100X)	5ml

Nucleosides (100X)

Thymidine	0.34mg/ml
Guanosine	0.7mg/ml
Adenosine	0.7mg/ml
Cytidine	0.7mg/ml

Nucleosides were made up to 50ml using ddH2O and filter sterilised.

(B) RPMI-1640

RPMI-1640	400ml
FCS	50ml
Penicillin / Streptomycin (10000µg/ml / 10000IU/ml)	5ml
Glutamine (200mM)	5ml

Appendix 3: Buffers and Solutions

(A) Phosphate Buffered Saline (pH 7.3)

5 tablets were added to 500ml ddH2O and autoclaved.

(B) Solutions for Agarose Gel Analysis of Fragmented DNA

The molarity indicated represents the final concentrations of reagents in the completed solution.

Tris-EDTA (TE) (pH 8.0)

Tris-HCL	10mM (pH 8.0)
EDTA	1mM (pH 8.0)

10X Tris Acetate EDTA (TAE) (pH 8.0)

Tris-base	24.22g (0.4M)
Sodium acetate	2.05g (0.05M)
Na_2EDTA	1.86g (0.01M)
ddH_2O	to 500ml

(C) HEPES Buffer for Annexin-FITC Binding Assay

HEPES/NaOH (pH 7.4)	10mM
NaCl	140mM
$CaCl_2$	5mM

(D) Solutions for Ca^{2+} measurement

HBSS (pH 7.4)

$CaCl_2.2H_2O$	1.3mM
$MgSO_4.7H_2O$	0.8mM
NaCl	140mM
$NaHCO_3$	4.2mM
$Na_2HPO_4.12H_2O$	1.3mM
Glucose	5.6mM

HEPES-buffered saline (pH 7.4)

NaCl	137mM
KCl	5mM
Na_2HPO_4	1mM
Glucose	5mM
$CaCl_2$	1mM
$MgCl_2$	0.5mM
BSA	1g/l
HEPES	10mM

(E) Solutions for Northern Blot Analysis

50X MOPS (pH 7.0)

MOPS	1M
$NaC_2H_3O_2$	0.25M
EDTA	0.05M

1M Sodium Phosphate (pH 7.2)

$Na_2HPO_4.2H_20$	89g
H_3PO_4 (conc.)	3-4mls, to pH
ddH20	to 1 litre

20X SSC (pH 7.0)

Sodium chloride	175.3g
Sodium citrate	88.2g
ddH2O	to 1 litre

Pre-hybridisation Solution

20X SSC	5ml
50X Denhardts	10ml
Formamide	16.5ml
50% Dextran Sulphate	10ml
20% SDS	1.25ml
50mM EDTA	200µl
10mg/ml ssDNA	500µl
ddH2O	6.55ml

Diethyl Pyrocarbonate (DEPC)-treated H_2O

500µl of DEPC was added to 500ml ddH_2O, mixed and left at room temperature overnight, followed by autoclaving.

Methylene Blue

Methylene blue was made up in 0.3M sodium acetate, pH 4.2.